1970

Studies On The Chemical Structure Of Phospholipids Isolated From Listeria Monocytogenes

Naim Kosaric

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NL-101(1/66)

STUDIES ON THE CHEMICAL STRUCTURE OF PHOSPHOLIPIDS ISOLATED FROM *LISTERIA MONOCYTOGENES*

by

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Department of Biochemistry

Submitted in partial fulfillment

of the requirements for the degree of

Doctor of Philosophy

Faculty of Graduate Studies

The University of Western Ontario

London, Canada

June, 1969

To my wife, Zekija, for all the years of help and understanding.

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This investigation was made possible by a grant from the Medical Research Council of Canada. The author expresses his sincere gratitude to this organization.

A C K N O W L E D G E M E N T S

Sincere appreciation and gratitude are expressed to Dr. K. K. Carroll who supervised this investigation, showing always a readiness to pass on his own ideas, knowledge and experience.

The author is also indebted to Dr. B. Serdarevich for his assistance in the decision to undertake these studies and for his valuable suggestions in NMR spectroscopy.

Appreciation is also expressed to Dr. W. L. Magee for his help and suggestions in the use of phospholipase A; to Dr. J. B. Strothers in helping with the interpretation of NMR spectra; and to Dr. R. Barel in assisting with the isolation and purification of the phospholipids.

Thanks are also extended to the members of the laboratory for their friendly co-operation when needed, and especially to Mr. H. E. Pedersen for his help in the isolation of phospholipids, preparation of Figures and his experienced technical suggestions.

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ABSTRACT

The phospholipids of Listeria monocytogenes were isolated by $CHCl_3$ and $CHCl_3$-MeOH extraction and were separated from non-polar components by acid-treated Florisil chromatography. They were further separated by preparative thin-layer chromatography and four main components were obtained. These components were analyzed chemically for ester groups, phosphorus, glycol groups and glycerol as well as by TLC and IR spectroscopy, and they were degraded by mild alkaline hydrolysis, acetic acid hydrolysis and by specific phospholipases. The degradation products obtained by mild alkaline hydrolysis were further purified by anion-exchange and Sephadex chromatography and analyzed chemically as well as by paper chromatography and NMR spectroscopy. The products of enzymatic degradation were also chemically analyzed as well as by TLC and gas-liquid chromatography, and the products of acetic acid hydrolysis were analyzed by TLC, paper and anion-exchange chromatography.

The structures for the three main bacterial phospholipids are proposed on the basis of the results obtained and the possibility of a phosphate triester structure for the fourth component is discussed.

The phospholipids found in this microorganism are all polyglycerophosphatides and three of them are represented by diphosphatidylglycerol, bis-phosphatidylglyceryl phosphate and phosphatidylglycerol structures. The fatty acids in these phospholipids are found to be non-randomly distributed with the shorter chain fatty acids being preferentially esterified to the β-position of the glycerols.

INTRODUCTION

Investigation of the lipids of Listeria monocytogenes was mainly initiated because of the interesting observations of the effects of this bacterium and its lipids on circulating white blood cells.

Although more than forty years have passed since the first description of the organism, there is still very little known about it and especially about the actual cause of the induced changes in the blood picture and the chemical composition of the bacterial lipids.

The first observations of Gram-positive rods in tissue sections of patients who died of an unknown disease, which is considered to be almost certainly a listeric infection, were made by Hayem in France in 1891 and by Henle in Germany in 1893. There were also some other reports (101) of a similar organism and infection in connection with it prior to the first isolation and description by Murray, Webb and Swann in 1926 (196).

These workers described a microorganism isolated from sick rabbits after an outbreak of infection in their laboratory colony in Cambridge. It was shown to be a small, Gram-positive motile rod, which was able to reproduce the disease in experimentally infected animals. In the course of the disease, a typical monocytosis was observed and because of this finding, the isolated microorganism was named BACTERIUM MONOCYTOGENES.

About the same time, in 1925, Pirie (209) isolated the same bacterium from the liver of gerbille in South Africa and noticed a

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About the same time, in 1925, Pirie (209) isolated the same bacterium from the liver of gerbille in South Africa and noticed a

marked necrosis of the liver in the infected animals. Because of this effect, he called the responsible organism LISTERELLA HEPATOLITICA. The name LISTERIA MONOCYTOGENES was later adopted and approved as the generic name in 1954.

Listeria monocytogenes is a member of the family Corynebacteriaceae, Order Eubacteriales. It is a small motile Gram-positive diphteroid-like flagellated rod with rounded ends, measuring 1.0 - 2.0 by 0.5 microns (231). There is evidence that the rod is capsulated (240).

Electron micrographic studies, reported by different investigators (101, 94, 95) show that the bacterium is surrounded by a thick cell wall (180 to 280 A) with three apparent layers. The outer and inner components are relatively thin with greater density whereas the middle component is normally thicker with lower density (95). The plasma membrane is a complex structure composed of three dense layers (15 - 30 A wide) which alternate with two light zones (average thickness 30 A). The cytoplasm is densely packed with granules of various sizes which are less than 100 A in diameter. The nuclear apparatus has the same general features as those found in other bacteria.

To mention some of the disorders associated with listeric infection in man, one can say that the bacterium attacks mostly the very young, sometimes even before they are born, or the very old. In the case of adults it is often superimposed on some other potentially grave disorder. In most cases the infection is associated with involvement of the central nervous system, causing meningitis as the

primary lesion and encephalitis, which if present results from extension of the process to the brain. Some other disorders are low grade septicemia, infectious mononucleosis-like syndrome, pneumonia, localized abscesses, conjunctivitis, mental retardation, habitual abortion, etc.

The mononucleosis, characteristic of listeric infection, attracted many investigators who had variable success in demonstrating the increase in circulating monocytes. This increase was shown by Murray to be as much as 6,000 times normal in rabbits (195).

The study of monocytosis developed after listeric infection gained very much in importance when Stanley, 1949 (245), discovered that a chloroform-soluble lipid, extracted from the bacterium, could produce such a response. He found that a monocyte producing agent (MPA) was extracted together with the lipids from Listeria monocytogenes and that it could be also extracted from the liver of infected rabbits. This was confirmed by Girard and Murray (96, 97).

Biological activity of lipid extracts from L.monocytogenes was also investigated in our laboratory (49). Besides the observed monocytosis, a marked depression in number of circulating lymphocytes was observed after injection of lipid extracts into mice. The overall response of the animals was dependent upon the origin of the bacterial strain and the temperature at which the organism was grown, as well as the sex and age of the infected animals (249). The maximum response (8% increase in monocytes and 40% decrease in lymphocytes) was observed with the lipids extracted from bacteria grown at lower temperatures (4°C) and tested in old female mice. It was also shown that the actual

MPA was associated with the lipids and extracted from the bacterium with the lipid solvents.

As the biological activity seemed to be mostly associated with the phospholipid fraction of the lipids extracted from the bacterium, the isolation, purification and determination of the chemical structure of phospholipids was of particular interest. A qualitative and quantitative study of the isolated phospholipids and development of new methodology in such a study will contribute to the knowledge of phospholipid distribution in this bacterium and help in clarification of this interesting activity of Listeria monocytogenes.

HISTORICAL REVIEW

I. THE BACTERIAL LIPIDS

Due to the lack of interest in the past and insufficient development of efficient methodology for the isolation and study of bacterial constituents, the systematic investigation of bacterial lipids is just beginning to develop on a full scale. The comparison of this development with the bacterial growth curve, as suggested by Kates (147) is possibly the best description of the trends in the past and the present status in this field.

The long period of time during which there was little activity and few techniques available, corresponds to the "phase of adjustment or the lag phase" which lasted till the late 1920's. At this time R. J. Anderson started his work on the lipids of the tubercle bacillus which activated the investigation in this field. The progression of this investigation was slow, lasting for about thirty years, and it could be compared with the "early growth phase". In this period the bacterial fatty acids were mainly investigated and little work was done on the nature of more complex lipid structures such as phosphatides and glycolipids. If we consider that the "logarithmic phase of growth" began in the middle 1950's when chromatographic techniques began to be introduced into lipid research, we are still at the lower portion of it progressing exponentially to the "stationary phase" which is still far from being reached.

Having realized the great importance of the bacterial world in the fundamental life processes on earth, and the ease and possibility

of growing and observing several generations of one microorganism in a relatively short period of time, the biochemist is more and more investigating bacterial processes and constituents. These processes, being in many cases similar or equal to the ones occuring in any living cell, can serve as models for study of basic biological interrelationships and rules of life in general. However, there are basic differences between the composition of mammalian and bacterial cells which have to be considered in interpreting and correlating the results obtained with the various types of cells.

The most radical differences between mammalian and bacterial cells in regard to lipid composition are manifested in sterol content and nature of fatty acids present in bacteria. Glycolipids and free fatty acids are also often present among bacterial lipids and the phospholipids in many strains have a low N/P ratio and are rich in inositol and carbohydrates.

The lack of sterols in bacteria was already recognized more than thirty years ago by Chargaff in C.diphtheriae (56), von Behring in E.coli (22) and by Hecht in Mycobacteria (117). The more recent results obtained by numerous investigators (12) confirm these previous findings, although in a very few cases sterols were reported to be present in small amounts. One example of such a report is that by Crowder and Anderson (70) who reported cholesterol in Lactobacillus acidophilus on the basis of a strong positive Lieberman-Burchard reaction for cholesterol. However, the authors suggested the possibility of cholesterol being derived from skim milk which was added to the culture medium. Dauchy and Kaiser (73) reported the

isolation of 5 mg of cholesterol from 100 g of dry E.coli but the isolated material, reported as cholesterol, did not seem to be pure according to its infrared spectrum. According to all these findings, the presence of cholesterol in bacterial lipids seems not to be confirmed.

Another major difference between mammalian and bacterial lipids is in the nature of the fatty acids. Bacterial lipids are often characterized by presence of branched chain acids, cyclopropane acids, and hydroxy acids. The branched chain fatty acids differ markedly in chain length and acids varying from 8 to more than 80 carbon atoms in the molecule have been reported (12). Some of those acids are discussed briefly.

One of the first isolated branched chain acids was tuberculostearic acid which was isolated by Anderson and Chargaff in 1929 from the tubercle bacillus (7). The acid was shown to be optically active and it showed biological activity in stimulating the proliferation of monocytes and epithelioid cells. The subcutaneous injection of this acid caused formation of tubercular tissue. Further investigations showed that it was present in lipids of both human and bovine strains of M.tuberculosis (13, 50, 51) and of M.phlei (12). The chemical structure of this acid was established by Spielman (242) in 1934 and it was shown to be composed of 19 carbon atoms represented by the following empirical formula: $C_{19}H_{38}O_2$. The branching is in the middle of the molecule so that the tuberculostearic acid is a 10-D-methyloctadecanoic acid. A series of branched chain acids have also been isolated by Gubarev and his collaborators (105) from the

lipids of Corynebacterium diphtheriae as for example α-corinnic acid ($C_{35}H_{68}O_2$) and β-corinnic acid ($C_{34}H_{66}O_2$). From the same bacterium Chargaff (56) isolated a monounsaturated branched chain acid called diphtheric acid ($C_{35}H_{68}O_2$). A very large group of branched chain fatty acids are C_{15} and C_{17} iso- and anteiso acids. The occurrence and distribution of these acids in bacteria will be more extensively discussed further in the text.

Another interesting acid in this series is lactobacillic acid $C_{19}H_{38}O_2$ which was isolated from Lactobacillus arabinosus in 1950 by Hofmann and Lucas (123). This acid contains a cyclopropane ring in the molecule represented as D-or L-cis-11,12-methyleneoctadecanoic acid. Other cyclopropane fatty acids with 17 and 19 carbon atoms have been isolated from numerous Gram-positive and Gram-negative bacteria (12).

Most of the hydroxy acids isolated from bacteria are β-hydroxy acids which almost all belong to the D-series (12). One of them is corynomycolic acid ($C_{32}H_{64}O_3$) isolated by Lederer and Pudles (164) from Corynebacterium diphtheriae in the amount of about 6% of the lipids present. The unsaturated homolog $C_{32}H_{62}O_3$ (Corynomycolenic acid) is also found in the same bacterium.

High molecular weight fatty acids have been isolated from every strain of Mycobacterium studied (12). All these acids are hydroxy acids with branched chains and more than 80 carbon atoms in the molecule. They were first isolated by Stodola et al. in 1938 (247) who proposed the formula $C_{88}H_{176}O_4$.

The glycolipids present in bacteria contain different sugars

and fatty acids which can involve a glycosidic or an ester bond between the lipid part and the sugar moiety. One of the representatives of the first group is the pyolipic acid, isolated by Bergstrom et al. in 1946 (27), from Pseudomonas pyocyanea. This acid was shown to be an L-rhamnoside of D-β-hydroxydecanoic acid with the sugar attached to the hydroxyl group of the acid. Esters of fatty acids and various sugars have been isolated from Corynebacterium diphtheriae by Gubarev et al. (105). Of particular interest are esters of trehalose as the "cord factor", discovered by Bloch in 1950 (31) was shown to be an ester of mycolic acid with D-trehalose. An ester of glucose and glycerol was isolated from Staphylococcus aureus by Macfarlane (174) and a diglycosyldiglyceride was found in the lipids of Listeria monocytogenes (49).

The free fatty acids found in bacteria do not generally represent a high proportion of the total lipids. In E.coli, Azobacter agilis and Agrobacterium tumefaciens their content is less than 10% as reported by Kaneshiro and Marr (145). In Sarcina lutea, Huston et al. found about 2% of free fatty acids (133). Free fatty acids were also found in the lipids of Listeria monocytogenes (49).

Almost all usual phospholipids are found in bacteria, such as phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, phosphatidic acid, phosphatidylglycerol, polyphosphatidylglycerols, phosphatidylinositides etc. (12). Phospholipids containing ether linkages were detected in Halobacterium cutirubrum (232). Plasmalogens were detected by Allison et al. (6) in Ruminococcus flavefaciens and by Bauman et al. (21) in Cl.butyricum. Sugar

containing phosphatides were reported in Lactobacillus acidophilus (71), Salmonella typhy (61) and several other bacteria. Amino acid containing phospholipids have also been detected in several bacterial species (12).

A. Lipid distribution in bacterial cells

The qualitative and quantitative distribution of lipids in different bacterial cells is a function of many factors which have an influence on the growth of bacteria as well as on the synthesis of individual lipid classes within the cell.

There is a great variation in lipid composition among the individual bacterial classes. Within the classes there is a variation in different orders, different families within an order as well as in different genera within a family. If we add to this that variation in lipid composition exists between Gram-positive and Gram-negative bacteria, and that even a particular strain of a single organism can be different, then one can realize the difficulty in generalizing about the results obtained in the study of bacterial lipids. However, some generalizations can be made, and an attempt is made to summarize briefly the different factors that influence the lipid content of bacteria, concentrating, where possible, to the Order Eubacteriales where Listeria monocytogenes is classified.

Several external factors can also influence the composition and distribution of bacterial lipids. Such factors are the composition of the culture medium, temperature of growth and age of the

culture. To these one can add such influences as pH of the medium, oxygen supply during growth, osmotic pressure and surface tension, which all can alter or modify the lipid composition of the cell.

1. Effect of chemical composition of media

High carbohydrate concentration in the medium increases the cellular lipid content and high nitrogen concentration decreases it. In the experiments with E.coli, Marr and Ingraham noticed (184) that cells grown in a chemostat limited by the concentration of a nitrogen source, showed a higher content of saturated fatty acids whereas at high nitrogen concentration this content was lower. Cells grown with a limitation in the concentration of glucose showed a slightly higher content of unsaturated fatty acids compared to the cells from the corresponding batch cultures without such a limitation.

In the study with the tubercle bacillus (12), it was found that the bacterium produced more lipids when glycerol was present in the medium compared to the medium without glycerol. Substitution of glycerol by glucose resulted in a decrease in total lipids as well as phosphatides.

The influence of acetate on lipids from human strains of the tubercle bacillus was studied by Barbier and Lederer (19). When acetate was added to the culture medium a decrease of lipid content was observed compared to the controls where no acetate was added. However, a marked increase in the total yield of bacteria (up to 180%) was noticed. It was also noticed that different strains of Mycobacteria behaved differently in the presence of acetate. There

was no growth with bovine strain and the saprophytic Mycobacteria showed an increase in lipid content as well as an increase in growth. Thorne and Kodicek (251) studied the effect of acetate and its replacement by mevalonic acid on growth and lipid content of Lactobacilli. The growth of bacteria was stimulated by acetate. Stimulation could be also demonstrated when acetate was replaced with mevalonic acid. Increasing glucose from 1% to 2% also resulted in a better growth even when acetate was not added to the medium.

Cho et al. (59) studied the effect of diphenylamine (DPA) on fatty acid composition in bacterial lipids from M.lysodeikticus, S.lutea and C.violaceum. The changes were manifested mostly in different quantitative distribution of fatty acids in cases with and without DPA added to the medium. The greatest differences were observed with M.lysodeikticus and S.lutea in the C_{17} anteiso acids which markedly increased after addition of DPA (from 2.6 to 34.6 and from 1.3 to 9.8% respectively). In the case of C.violaceum. the greatest difference was a big increase of C_{20} acid (from trace to 10.4%). C_{15} anteiso acid showed a slight decline in all cases when DPA was added.

The influence of different culture media on lipid composition of C.diphtheriae was studied by Cmelik (60). Dagley and Johnson (72) investigated the lipid content in Bact.coli in the presence of various amounts of glucose and acetate. When glucose was kept constant and 0.3 M acetate was added to the medium, the lipid content gradually increased as a function of the quantity of added acetate. In other experiments, where acetate was kept constant, the increase

in glucose caused a decrease in total lipid produced by the organism. Kates, Sehgal and Gibbons (156) studied the influence of salt concentration on lipid composition of Micrococcus halodenitrificans. No qualitative changes could be detected in the phospholipids and fatty acids when the cells were grown in media containing different concentrations of sodium chloride. However, the cells grown at low salt concentration (0.6 M) contained considerably more unsaponifiable matter than the cells which were grown at optimum concentration of sodium chloride (1.0 M).

White and Frerman (270) studied the growth of Staphylococcus aureus under aerobic and anaerobic conditions. The composition of fatty acids was followed under both conditions, and it was found that the proportion of the acids was not very significantly changed. The main fatty acid in phosphatidylglycerol and diphosphatidylglycerol in this organism was found to be C_{15} anteiso acid and its relative amount increased slightly in cultures grown under aerobic conditions.

There is also another interesting observation that microorganisms in general can take into their cells and further metabolize many lipids which are originally present in the medium. The fatty acid content of bacterial cells can also be regulated qualitatively and quantitatively by varying fatty acid content in the medium (122, 124, 221).

An increased oxygen supply tends to increase the total lipid content as well as the percentage of saturated lipids in the cell. Whether the first effect is due directly to oxygen or is just an effect of the aging process due to more rapid growth is not yet

completely investigated. It is difficult to say whether osmotic pressure, surface tension and pH are directly related to lipid levels in microorganisms. Many differences in lipid content can, under the variation of these factors, be just a reflection of the alteration in growth rates and metabolism of the microbial cells.

2. Effect of growth temperature

The effect of temperature on bacterial lipid composition is especially well demonstrable in the case of fatty acids. At low temperatures microorganisms tend to increase their content of unsaturated fatty acids while at higher temperatures these acids are decreased. The effect was well demonstrated by Marr and Ingraham (184) who noticed that the proportion of unsaturated fatty acids of E.coli continuously decreased as growth temperature was increased. The increase of unsaturated acids at lower temperatures was also noticed by Rose (222).

Listeria monocytogenes, grown at different temperatures (Carroll et al. - unpublished data) also shows an alteration in fatty acid composition. At lower temperatures, there is a tendency to produce more shorter chain fatty acids (C_{15} anteiso and shorter), whereas at higher temperatures the proportion of longer chain acids (mainly C_{17} anteiso acid) is increased.

The effect of temperature on phospholipid composition has scarcely been investigated and probably the main differences are due to the different fatty acids rather than to alteration in the basic structure of the phospholipid.

3. Effect of age of the culture

Another factor which is able to cause large variations in the lipid content of various bacterial species is the age of the culture. Generally, the total lipids and the degree of saturation increase with the age of the bacterial culture (147).

Asselineau (14) studied the lipid content of the tubercle bacillus in relation to age of the culture. The quantity of total lipids was about 7 - 10% in the young cultures (5 days) compared to 20 - 22% in the older cultures (42 days). The content of free fatty acids was also greater in the old cultures. However, the phosphatide content did not change with the age of the culture.

Law and his collaborators (162) observed that the formation of cyclopropane acids in A.tumefaciens was highly dependent upon the age of the organism. The formation of the acid was nearly linear and increased with age even after growth of the cells has ceased. A similar effect was observed in E.coli which also showed an increase in fatty acids and total lipids with the age of the organism. Kanfer and Kennedy (146) found that the content of phosphatidylglycerol was much greater in rapidly growing cells of E.coli than in cultures in stationary phase. The amino acid esters of phosphatidylglycerol have been reported to accumulate in the stationary growth phase of S.aureus (147). The lipid content of Mycobacterium phlei was shown to be minimal during exponential growth (11).

According to all these findings, it is very important, in order to obtain a defined reproducible lipid content in a microbial culture, to maintain strictly all the mentioned variables, to keep

the conditions as constant as possible and to stop the growth at a defined age of the culture. This is of particular importance in the case of repeated experiments or preparation of great quantities of cells which are pooled together after harvesting in different batches.

There is also another factor which should be considered and which is particularly important in the case of pathogenic bacteria. Very often these microorganisms show different growth characteristics and different lipid compositions when grown *in vivo* and *in vitro*. Differences in other characteristics such as biological activity, overall cell composition, etc. are also markedly influenced by this factor and have to be taken into consideration.

4. Correlation of lipid composition with different taxonomic characteristics

As already mentioned, the lipid composition varies to a great extent in different bacterial classes. The first attempt to classify bacteria according to their lipid composition was made by Abel *et al.* in 1963 (1). They showed that there existed a correlation between taxonomic characteristics and the fatty acid composition in different bacterial species. This new criterion was of particular value in the troublesome classification of bacteria and the study of their evolution. In general, members of the same family have qualitatively similar fatty acid composition, whereas the composition within different families differs. Comparing different orders, one can clearly distinguish *Eubacteriales* from *Actinomycetales* because *Actinomycetales*

have much more complex fatty acid, phosphatide and neutral lipid components. An exception to this rule is the family Corynebacteriaceae which contains very complex lipids.

There are, according to Kates (147), two basic differences which could be correlated with different bacterial characteristics: differences in fatty acids and differences in phospholipids and other lipid classes. The differences could be attributed to the difference in Gram-stain and the family affiliation.

Concerning the fatty acid composition, there is a clear distinction between Gram-positive and Gram-negative bacteria in the order Eubacteriales. The Gram-positive bacteria are characterized by a high proportion of odd-numbered, branched-chain iso or anteiso acids and relatively low amounts of straight-chain saturated or unsaturated acids. The Gram-negative bacteria have high proportions of normal, even-numbered saturated and unsaturated acids and odd-numbered cyclopropane acids.

However, some of the bacteria which are Gram-positive have fatty acid spectra resembling those of Gram-negative bacteria. As an example, Corynebacteria, which are Gram-positive, do not contain appreciable amounts of simple branched-chain acids but have C_{20} and C_{28} saturated and unsaturated acids and complex hydroxy branched C_{32} acids.

The correlation of fatty acids on the basis of family affiliation is even more regular within the bacterial families, taking into consideration the effect of age, culture and temperature. Members of the family Corynebacteriaceae were found to have a high proportion

of complex, branched-chain, high molecular weight acids, some of which were hydroxylated (147). The lower molecular weight acids are normal saturated C_{14} - C_{24} acids and unsaturated C_{14}, C_{16}, C_{21}, C_{22-24} and C_{28} acids. Listeria monocytogenes, with its unusual acid content, although classified according to its taxonomic characteristics in the family Corynebacteriaceae, differs from this general rule in having as the major acids C_{15} and C_{17} anteiso acids with some minor quantities of C_{14} and C_{16} normal and iso acids (49, 214, 234).

The correlation of phosphatide composition with the Gram-stain of the cells is, according to scarce data on phosphatide composition, a more difficult task. However, there is some indication that such correlation exists. The phosphatides of Gram-positive bacteria do not generally contain phosphatidylethanolamine. They are rich in acidic phospholipids of the phosphatidylglycerol and diphosphatidyl-glycerol type as well as in amino acid esters of phospholipids. They also contain considerable amounts of glycolipids (glycosyl diglycerides) and very little or no lipopolysaccharides. The Gram-negative bacteria, on the contrary, have high proportion of nitrogen-containing phospho-lipids such as phosphatidylethanolamine. They do not contain considerable amounts of glycolipids and are characterized by a high proportion of lipopolysaccharides (147). These observations are of fundamental value for distinguishing between these two groups but there are many exceptions and some Gram-positive bacteria have been found to contain phosphatidylethanolamine and some Gram-negative glycolipids. However, one can generalize that Gram-positive bacteria have less nitrogenous containing phospholipids and that the N/P ratio

in their phospholipids is low compared to Gram-negative classes. These differences in phospholipid classes are found to be due to differences in lipid composition of the subcellular structures. The lipid composition of the bacterial cell is not uniform throughout the cell mass but is rather specific for different substructural units such as cell wall and cell membranes. The quantity of lipids present in the cytoplasm is small and the far more important locations are in the cell wall and the cytoplasmic membranes.

5. Cell wall lipids

The cell wall which surrounds the cytoplasmic membrane and maintains the rigid structure of the cell is a complex structure. It differs in Gram-positive and Gram-negative bacteria (147) being multilayered in the case of Gram-negative bacteria but generally not in Gram-positive bacteria as seen by examination of the cells under the electron microscope.

These two groups of bacteria differ markedly in the lipid content of their cell walls. Gram-negative bacteria have a high content of lipids (up to 26%), while Gram-positive have very little or no lipid. Another difference is that walls of Gram-positive bacteria are rich in peptidopolysaccharides and glycopeptides, whereas the cell walls of Gram-negative bacteria have less mucopeptide and large amounts of protein and polysaccharide (229).

The different layers of the cell wall have also been investigated (186, 265). It was found in E.coli that the outermost layer (about 60% of the cell wall weight) consisted of lipoprotein, the

middle layer (12%) of lipopolysaccharide and the inner "rigid layer" (21%) of mucopeptide complex containing small amounts of lipid (5 - 8%).

Complex cell walls are also present in some Gram-positive bacteria. Corynebacteria, for example, have complex cell walls which contain appreciable amounts of lipids. A complex cell wall was also observed in L.monocytogenes by Ghosh and Murray (95). The distribution of lipids in Corynebacterium diphtheriae was investigated by Alimova (5) who concluded that the cell wall had an outer layer of liponucleoprotein containing trehalose esters of unsaturated C_{16} and C_{20} acids and free fatty acids. The inner layer was composed of lipoglycoprotein containing trehalose esters of unsaturated C_{16}, C_{20} and C_{22-24} acids, small amounts of free fatty acids, mucopeptides, proteins and polysaccharides.

Marr and Kaneshiro (185) analyzed the lipids of the cell envelope of Azobacter vinelandii. The total lipid content was found to be 28%, consisting of 10 - 20% neutral lipids and 70 - 75% of phospholipids. The main components of the neutral lipids were free fatty acids (more than 50% palmitoleic acid) with traces of glycerides. The polar portion was composed mainly of phosphatidylethanolamine, containing myristic, palmitic, palmitoleic and C_{18} monoenoic acids.

6. Lipids in cytoplasmic membranes

a. Organization of membranes

The highly organized membrane network of living cells and its

recognized function in vital processes, puts these cell structures in a particularly favorable position for biochemical research. Besides its function of separating different compartments in the cellular mass and its regulation of the translocation and permeability of chemical substances, a great deal of the enzymatic machinery of the cell is organized on membranes, governing in this way the whole metabolism of the cell.

The presence of lipids in the membranes was already suggested in the 19th century by Overton. Much evidence has been accumulated since that time which supports the idea that lipids and especially phospholipids play a very important role in the function of the cellular membranes. Highly organized with proteins, the phospholipids function in such vital processes as transport across the cell membrane, carrying cations and anions and creating an osmotically active barrier which enables the functioning of the whole intra- and extra-cellular system in the living organism. Their involvement in many other essential functions of the cell, such as swelling and contraction of mitochondria, amino acid transport, protein synthesis, lipid and sugar transport, etc., cannot be sufficiently emphasized.

The exact arrangement of lipids and proteins in the cellular membranes is not yet known. Several models have been proposed and the classical "unit membrane", proposed by Robertson in 1959, seems still to be a good scholastic representation of a membrane structure. In this model, the membrane is represented by a lipoprotein complex, where the lipids are arranged in a bimolecular layer, about 40 - 60 A thick. The lipids are covered by a protein layer (10 A), from both

sides, forming in this way a three layered "unit membrane". The polar groups on the lipids are thought to be oriented so that they are in direct contact with the corresponding groups of the protein, being held together preferentially by ionic bonds. However, as more evidence accumulated, this model was subjected to criticism and newer models have been proposed. As the membranes are the site of most or in some cases all phospholipids in the cell, a short summary of the present concepts of membrane structure and function is here presented.

As already mentioned, the membranes should not be viewed only as the structures which separate different compartments in the cell, but rather as specific sites of many metabolic processes and molecular biosynthesis. In this light, maybe the best definition of a cellular membrane is the one given by Korn (160) that "membranes are aggregates of controlled, directional metabolic processes". As these processes are different in different cells, the membranes are different in chemical composition, in enzymatic content and metabolic function. Therefore, there would be no justification for a complete identity of different cellular membranes.

In the newer membrane models, the protein is considered to be the fundamental unit of structure to which lipids are added in specific arrangements which are dictated by the structure of the protein. The result of this interaction would be lipoproteins which could then interact with each other and form a membrane in which specific enzymes are incorporated. As visualized by Korn and suggested by Jardetzky (138), a protein-bound solute molecule could be moved from one side to the other by an allosteric transformation

of the protein which is by many investigators suggested to be predominantly in the α-helical form (160). The bonding between lipid and protein is mainly hydrophobic in nature and there is no conclusive evidence for ionic interactions in the membrane (103, 263). As suggested by Wallach and Gordon (263), lipid and protein association must be via weak interactions such as charge-charge interactions, hydrogen bonding, London - van der Waals forces and hydrophobic bonding. However, ionic interaction takes place between acidic phospholipids and basic proteins such as cytochrome c(103).

Specific transport mechanisms are suggested for the transport of solutes through the membrane. In cases where such transport mechanisms do not exist, the lipids would provide the basis for the relative impermeability of the membrane. Korn also suggested a special role of lipids in the membrane, which is manifested by an influence on catalysis.

Based on the above mentioned principles, several new models of membranes have been proposed. Green et al. (102) have presented electron microscopic evidence for membrane formation by repeating units of the individual complexes of electron transfer chain and different membrane basepieces. The hydrophobic bonds link one repeating unit to another and they could be ruptured by bile salts. Association of the lipid-containing repeating units leads to formation of the membrane. When lipids were removed, a bulk phase was formed in which enzymic activity could not be demonstrated. After reintroduction of lipids into the extracted repeating units, the capability of membrane formation was restored. The membranes which

were formed by this process are visualized as hollow spheres in which the periphery is made up of fused repeating particles, one particle thick. In this way the membrane represents a "molecularized" state of repeating units and each unit is suggested to be in equilibrium with solute molecules in the medium.

Another model has been proposed by Wallach and Gordon (263). In this model, the membrane peptide is located on both of the membrane surfaces and also within the nonpolar interior of the membrane. The penetrating protein segments are helical rods similar to the H helix of hemoglobin. The apolar amino acids are located on the external surfaces of the subunit assemblies and represent specific binding sites for the membrane lipids. The head groups of those lipids are suggested to participate in polar associations with surface located protein side chains. The tight binding of lipids with the proteins in the membrane is suggested to be analogous to the heme-protein interactions in hemoglobin. Polar amino acids are so distributed that their side chains lie in the membrane surfaces or are localized around the central axis of each subunit, producing in this way aqueous channels which penetrate the membrane. The complex structure of the proteins in the membrane is strongly dependent upon their association with lipids and represents a state of lowest free energy and maximal entropy of the protein-lipid-water system. The contact between proteins and lipids as well as protein subunits is envisaged to occur on both polar and apolar regions of the membrane.

Other models, suggested by Lenard and Singer (165), Steim and Fleischer (246) and Wallach and Zahler (264), emphasize protein-protein

interactions as the basic forces which hold membranes together. The amino acid side chains and the fatty acyl groups of the lipids provide an internal hydrophobic environment where appreciable α-helical regions of the proteins are located.

New models will probably be suggested as new techniques develop and more information accumulates on the structure and function of cellular membranes. The proteins and lipids are definitely the essential parts of membranes but there is a real possibility that the difference between various membranes is so great that no generalization concerning membrane structure and function can be made. In this light, the membranes "cannot be usefully described by one unifying model" (160).

b. Membrane lipids

Lipid and protein make up at least 70% and in some cases, almost the whole weight of the cytoplasmic membrane (147).

Although there is not much information available on the lipid composition of the membranes of Gram-positive bacteria, deductions are easily made by analysis of the total cellular lipids, taking into account the fact that almost all of the lipids present in the cell are localized in the membrane (147, 259), with little or no lipid in the cell wall. In general, in Gram-positive bacteria, the lipids make up 15 - 30% of the dry weight of the membrane preparation. The rest is almost entirely protein, with 1 - 20% carbohydrate present in some cases (166).

An extensive study of the composition and structure of the membrane from Listeria monocytogenes has been reported by Ghosh and

Carroll (94). The crude membrane preparation obtained after osmotic lysis of protoplasts was extensively washed with NaCl and $MgCl_2$ in Tris buffer, resulting in a pure membrane preparation without contamination with cytoplasmic material. In this preparation 55 - 60% of protein and 30 - 35% of lipid was found. There were also minor quantities of RNA, DNA and carbohydrate. The lipids were composed of 80 - 85% phospholipid and 15 - 20% neutral lipid, the phospholipid being mainly of the phosphatidylglycerol type.

It has not yet been possible to obtain a pure membrane preparation of Gram-negative bacteria, where the demonstration of a separate cytoplasmic membrane is only possible by electron microscopy. However, it is likely that a considerable proportion of the cellular lipids of these organisms is located in the cell envelope, together with lipopolysaccharide, which seems to be localized at the same site (203).

B. Biosynthesis of bacterial lipids

The current concept of biosynthesis of bacterial lipids and especially phospholipids and fatty acids associated with them is briefly discussed. In general, the established pathways for the biosynthesis of lipids in mammalian and plant worlds are applicable to the microbial world as well.

1. Biosynthesis of fatty acids

There were many attempts in the past to explain the biosynthesis of the lipids, and particularly fatty acids, on the basis of a close

interrelationship between the degradative and synthetic processes in the metabolism of the biochemical constituents. The fact that various reactions of the β-oxidation system of fatty acid degradation are reversible, strengthened such an approach with Lynen being one of its first supporters.

However, in the early 1960's, it was demonstrated by Wakil (261) that the biosynthesis of fatty acids was a more complicated process than previously postulated.

Evidence for the presence of two distinct systems for the synthesis of fatty acids was presented: one which seems much more widely distributed in living organisms and the other, less important, which involves the reversal of several reactions of the β-oxidation system of fatty acids.

The first pathway was shown to be the major pathway in the biosynthesis of saturated, normal chain fatty acids with even numbers of carbon atoms in the molecule. It was extensively studied in a number of laboratories (32, 169, 257, 260, 261) and was found to be composed of a cycle of repeating reactions, leading to the final product--a long chain fatty acid. The basic requirement for the overall reaction is acetate, present in its active form as acetyl CoA which could be converted to fatty acid in the presence of ATP, Mn^{++}, HCO_3^- and NADPH. The actual condensing unit, however, is shown not to be acetyl CoA itself, but rather its carboxylated derivative, malonyl CoA (261). It was produced by a two-step reaction from acetyl CoA catalyzed by a specific (Biotinyl) acetyl-CoA-carboxylase enzyme. The presence of acetyl CoA was found to be absolutely necessary for

The initiation of the reaction and the condensation in the subsequent step with malonyl CoA. However, further condensation steps are not associated with the CoA derivative as such but rather with a heat stable protein cofactor-acyl carrier protein (ACP), isolated and characterized by Majerus et al. (181).

The second pathway does not seem to play an important role in biosynthesis of saturated fatty acids in bacteria. It possibly plays a role in the elongation of existing fatty acids by addition of two carbon units at a time (261).

a. Iso and anteiso fatty acids

It has been shown by numerous investigators that a variety of Gram-positive bacteria contain branched chain fatty acids comprising in some cases more than 50% of the total fatty acids present in the organism (148). These fatty acids are mostly C_{15} and C_{17} iso and anteiso acids. The iso forms are chemically 13-methyltetradecanoic and 15-methylhexadecanoic acids, respectively, while the anteiso-forms of these acids are represented as 12-methyltetradecanoic and 14-methylhexadecanoic acids. These acids have been reported in bacteria by Akashi and Saito (4), Macfarlane (172), Kaneda (141, 142, 143, 144), Urakami and Umetani (255), Tornabene et al. (252), Moss et al. (194), Serdarevich and Carroll (234), Raines et al. (214), and others.

Urakami and Umetani found about 80% of C_{15} and C_{17} anteiso acids in the total acids of phospholipids from Bacillus natto. The proportion of the anteiso to the iso form increased with the time of incubation. Tornabene, Bennet and Oro studied the distribution of

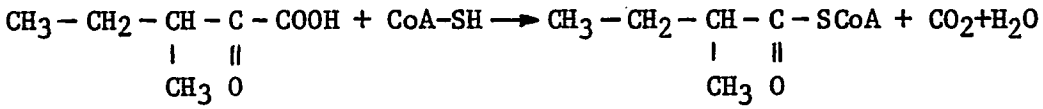
fatty acids in Sarcina lutea as a function of different growth media and found predominantly C_{15} anteiso acid which in a defined medium amounted almost to 70% of the total fatty acids present. The fatty acids in Corynebacterium acnes (194) were also mainly C_{15} and C_{17} anteiso acids. Extensive study of fatty acid composition in different bacterial species was done by Kaneda who analyzed more than ten different species of the genus Bacillus. They all have been shown to produce predominantly branched chain fatty acids (C_{15} and C_{17} iso and anteiso acids). It was also shown that the fatty acid distribution varied in organisms grown on different media.

It is interesting to mention that the iso and anteiso branched chain fatty acids have been also found in some other sources besides bacteria. Weitkamp (266) found C_{15} and C_{17} anteiso acids in wool fat and wool wax. The C_{17} anteiso acid was also isolated from mutton fat (110). Hradec characterized the 14-methylhexadecanoic acid as the acid esterified to cholesterol in carcinolipin (129). Branched chain fatty acids have also been isolated from human blood (104). Because of the structural resemblance of these fatty acids to isoleucine and leucine, it was suggested by Lennarz (167) and by Horning et al. (127) that the biosynthesis of these acids might be linked to the amino acids.

Starting with isoleucine as the precursor, the following reaction sequence is proposed for the formation of 12-methyltetradecanoic acid:

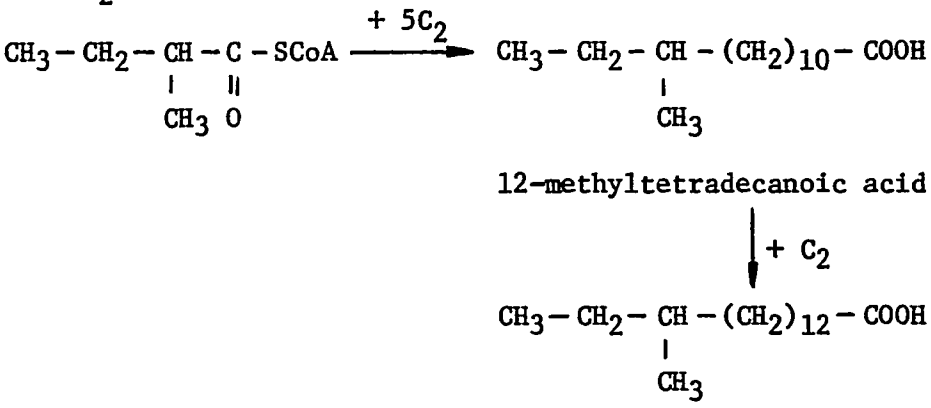
isoleucine 3-methyl,2-ketopentanoic acid

The intermediary product, 3-methyl,2-ketopentanoic acid can react subsequently with CoA giving a CoA derivative, 2-methylbutyryl CoA, with decarboxylation of the pentanoic acid, according to the following scheme:



2-methylbutyryl CoA

The methylbutyryl CoA can, according to the malonyl CoA pathway, give rise to the anteiso branched chain acid by successive elongation with two CH_2 units.



12-methyltetradecanoic acid

14-methylhexadecanoic acid

The iso C_{15} and C_{17} branched chain acids can be produced by the same pathway starting from leucine (148).

The presence of minor quantities of iso-C_{14} and C_{16} acids in the lipids of L.Monocytogenes has been reported by Serdarevich and Carroll (234). Valine or isobutyric acid can serve as precursors for these acids.

2. Biosynthesis of phospholipids

As already mentioned, phospholipids compose the bulk of the

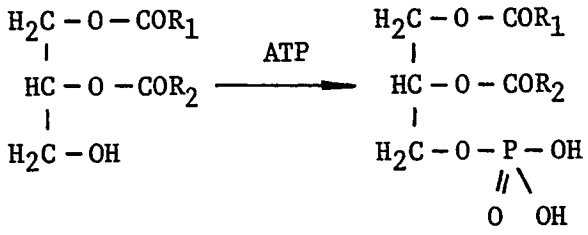
lipids present in bacterial cells. Their biosynthesis has been extensively studied by Kennedy and his group. The key substance, which plays the central role in the biosynthesis of phospholipids is shown to be phosphatidic acid, which can by various pathways give different phospholipids according to the general scheme proposed by Kennedy and his associates (159). The scheme is represented in Fig. 1.

There are three major pathways known for the biosynthesis of phosphatidic acid (148):

1. Stepwise acylation of α-glycerophosphate with acyl CoA demonstrated by Kornberg and Pricer (161) with the enzyme acyl CoA L -α-glycerophosphate acyl transferase:

$$\alpha\text{- GP} \xrightarrow{\text{acyl CoA}} \text{Lyso-PA} \xrightarrow{\text{acyl CoA}} \text{PA}$$

2. Phosphorylation of a diglyceride with ATP catalyzed by diglyceride phosphokinase:



3. Phosphorylation of a monoglyceride by ATP with lysophosphatidic acid as the product. The enzyme involved in this reaction is monoglyceride phosphokinase which was isolated from brain by Pieringer and Hokin (210). The lysophosphatidic acid, produced in this way, can further be acylated with fatty acyl CoA and lysophosphatide Acyl CoA transferase (211):

$$\text{Monoglyceride} \xrightarrow{\text{ATP}} \text{Lyso-PA} \xrightarrow{\text{acyl CoA}} \text{Phosphatidic acid}$$

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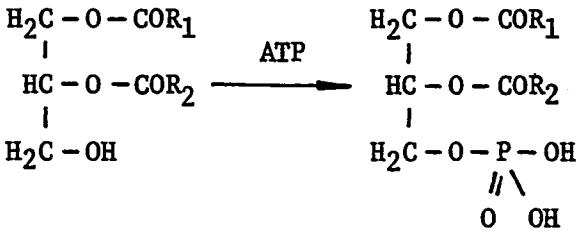

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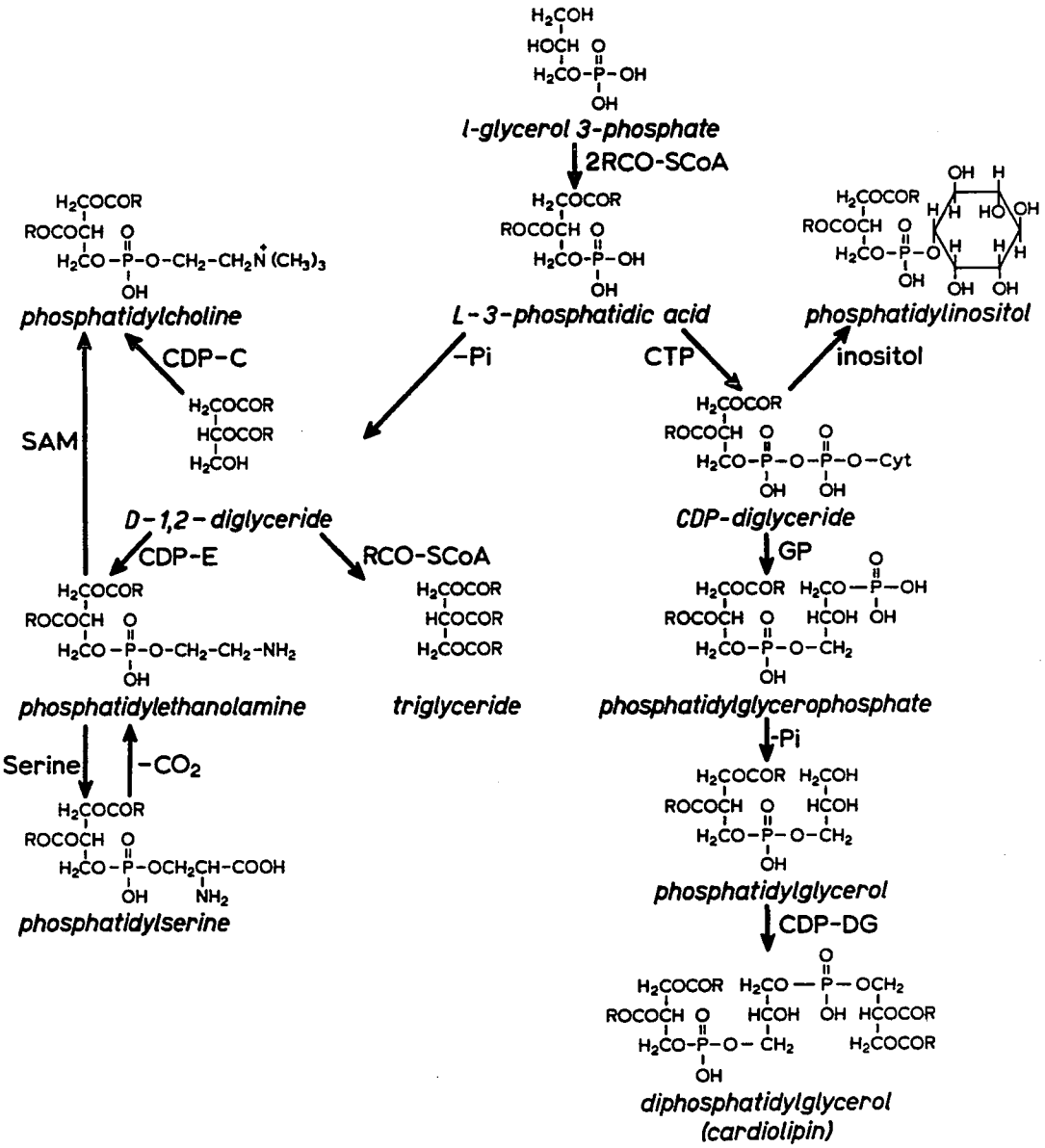
F I G U R E 1

Phospholipid interrelationships

The interrelationships of natural phospholipids according to Kennedy (152) are illustrated.

PHOSPHOLIPID INTERRELATIONSHIPS



Although the particulate enzymes for the three pathways were originally isolated from mammalian tissue, there have been several demonstrations of the involvement of these systems in microorganisms (148). According to the accumulated data, it seems that the first pathway is general in the bacterial world.

The other requirement for the biosynthesis of phospholipids is cytidine diphosphate diglyceride, which is synthesized in animal systems from phosphatidic acid and CTP, catalyzed by phosphatidic acid cytidyl transferase. The requirement of cytidine triphosphate for this reaction is highly specific (158) and it cannot be replaced by any other nucleotide.

According to this scheme, CDP - diglyceride is an important intermediate in the synthesis of phosphatidyl inositol as well as cardiolipin, the ultimate products in the synthesis of acidic glycerophosphatides.

The first evidence for the formation of CDP - diglyceride came from Agranoff, Bradley and Brady (2). However, due to the small quantities of products, they did not succeed in isolation and adequate characterization of this important intermediate.

Paulus and Kennedy (207) were able, in 1958, to demonstrate a specific requirement of CTP as cofactor for the conversion of L-α- glycerophosphate p^{32} to phosphatidyl inositol, which in this way differed from the reactions leading to the formation of phosphatidyl-ethanolamine and phosphatidylcholine. Their synthesis, as mentioned, proceeded through the CDP derivatives. After the successful synthesis of the cytidine diphosphate diglycerides by the same group (208),

it was possible to prove this proposition. In the proposed pathway, the intermediate in the reaction which is formed after enzymatic dephosphorylation of the phosphatidylglyerophosphate is shown to be phosphatidylglycerol. This compound, discovered in 1958 by Benson and Maruo (24) in Scanedesmus cells and isolated by Macfarlane (173) from Micrococcus lysodeikticus, was suggested as an intermediate in the synthesis of cardiolipin and other polyglycerophosphatides. The evidence for the last step, condensation of phosphatidylglycerol with CDP-diglyceride, came from the work of Stanacev, Chang and Kennedy (243). They found that the conversion of phosphatidylglycerol to cardiolipin was largely dependent on added CDP-diglyceride and proceeded as previously postulated and represented in Fig. 1.

C. Biological properties of bacterial lipids

As the biological properties of Mycobacteria have been studied much more intensively than the properties of other bacterial classes, their biological influence is discussed together with some of the known actions of other bacterial species.

One of the first experiments which demonstrated the biological activity of lipids, and particularly phospholipids, was performed by Sabin, Doan and Forkner (228). They injected subcataneously or intraperitoneally a phosphatide fraction obtained from the tubercle bacillus into rabbits and observed the formation of granuloma with parallel transformation of monocytes into epithelioid cells and the formation of giant cells by atypical mitosis of a single cell. However, the fractions which were examined in these first experiments

were not fully defined so that it was difficult to correlate the activity with any specific lipid compound. In some further experiments done by Sabin (227) it was concluded that the activity of the phosphatide fraction was due to the phthioic acids present in this group of lipids.

After these "preliminary experiments", numerous branched chain fatty acids, newly synthesized or isolated from the tubercle bacillus were tested for their biological activity. It was demonstrated in these experiments (12) that the synthetic acids were as active as the natural ones, although it was not possible to demonstrate a compound with the maximum activity.

It was also found that other types of lipids can cause the formation of granuloma. These were mainly the mycolic acids (92) which could cause a necrotic reaction and formation of giant cells and can also influence the persistance of lesions induced by the tubercle bacillus. The development of granuloma was also observed after injection of lipids isolated from other microorganisms such as Colon bacilli (217), Streptococci (241), Aspergillus fumigatus (244) and Listeria monocytogenes (96).

Besides their influence on the normal development of the cell as such, lipids extracted from different microorganisms have also been shown to have an influence on enzyme action. The inhibitory effect of unsaturated fatty acids on trypsin and some proteases was observed by Jobling and Petersen (140) as early as 1914. There were also some later observations of inhibition of enzymes by fatty acids, as for example in the case of lecithinase (91). This activity seemed

to be correlated with the configuration and molecular weight of the acids. It was also reported that the unsaturation of the acid was not a requirement for this activity.

Another active constituent of the lipids, the "cord factor" could also be demonstrated (157). It had the ability to decrease the activity of several enzymes present in the liver. The enzymes were all dehydrogenases with some of them having NAD as cofactor.

1. Action of lipids on leucocytes

The first observation of the different behaviour of leucocytes in the presence of fatty acids was demonstrated by their diminished chemotactism for starch granules. Performing these experiments with lipids from the tubercle bacillus Fethke (86) draws the conclusion that the fatty acids with more than ten carbon atoms in the molecule were responsible for this phenomenon. Other microorganisms such as Chigella dysenteriae and E. coli (12) have been found to stimulate the phagocytic activity of rabbit leucocytes. The lipid extract of Corynebacterium ovis is also capable of provoking degeneration and death of sheep and guinea pig leucocytes.

The biological activity of the lipids isolated from Listeria monocytogenes has already been mentioned. During the experiments with lipid extracts from a strain isolated by Stanley from a human case of meningitis (strain 42), it was noticed that the experimental animals (mice) showed a decrease in circulating lymphocytes after intraperitoneal injection. However, this response could not be observed with all strains of this bacterium, which in some cases did

not show any biological activity or the activity was manifested only in monocytosis of the infected animals (249). The reasons for such different response and the exact nature of the active substance (or complex) is not yet known.

2. Toxicity

A single toxic factor has been demonstrated in the tubercle bacillus. It was called "cord factor" and was discovered in 1950 by Bloch (31) and identified in 1956 (199) as trehalose-6,6'-dimycolate, a waxy material with high toxicity for mice. It acts selectively on capillaries, destroying their fine structure.

Another toxic lipid was found in Corynebacterium ovis, a diphtheroid bacillus which is pathogenic for sheep. The leucocytes which engulf some of these bacilli undergo rapid degeneration and die. The same effect on leucocytes could be demonstrated (46) with a lipid extract obtained from this microorganism. The toxicity of water-soluble polysaccharides which were isolated from Gram-negative bacteria has been shown by Westphal et al. (269) to be due to a "Lipide A", an endotoxin of E. coli 08. It is strongly pyrogenic and produces marked leucopaenia.

A toxin of Listeria monocytogenes was demonstrated by McIlwain and Barnes (191). The toxin was produced by the microorganism in vivo and its effect was tested on chinchillas. Cells of L. monocytogenes, placed in sealed diffusion chambers, were surgically implanted in the peritoneal cavity of the experimental animals. The time to death of the animals was investigated and it was found that the animals died

earlier than when the organisms were administered by intraperitoneal inoculation.

3. Antigenicity

The antigenic properties of phospholipids have long been recognized, dating from the first observations back in the 1920's when Negre and Boguet (198) showed that a methanol extract of the tubercle bacillus, previously treated with acetone, possessed antigenic properties. It could fix the complement in sera containing antibodies against the tubercle bacillus and it had a high proportion of phospholipids.

The purification of phospholipid antigens has been extensively studied by Pangborn (205, 206) who was able to isolate two active phospholipopolysaccharides, one of which contained 1.7% of phosphorus, 10.5% of inositol and 50% of mannose, being probably a pentamannoside of phosphatidylinositol.

The alcohol-soluble lipids isolated from Corynebacterium diphtheriae have been shown by Hoyle (128) to fix the complement of rabbit immune sera. The antigens of this organism also produced agglutination of the chicken red blood cells. This property has also been ascribed to phospholipids. From the same microorganism, Hara (111) was able to make a cardiolipin-type preparation which reacted with syphilitic serum as well as with the phosphatide preparations from M. tuberculosis. The reactivity of different synthetic cardiolipin analogues with syphilitic sera was studied by Inoue and Nojima (135). They suggested that the reactivity of cardiolipin with

Wassermann antibody was attributed to the presence of a free hydroxyl group, two phosphate groups and the diglyceride moiety.

II. PHOSPHATIDYLGLYCEROLS

As mentioned previously, Gram-positive bacteria contain a high proportion of phosphatidylglycerols but these compounds have not been investigated as extensively in the past as other phospholipids. The reason for this is mainly because of the difficulties associated with isolation and their relative instability during treatment. The lack of characteristic functional groups which could be followed during investigation complicated the work done on their isolation, purification and investigation even more.

Phosphatidylglycerols in general have a common characteristic in that they are all acidic in nature. They are all fatty acid esters of one or more molecules of glycerol which are also esterified to phosphoric acid, forming in this way a series of interrelated compounds with three main components: fatty acids, glycerol and phosphate.

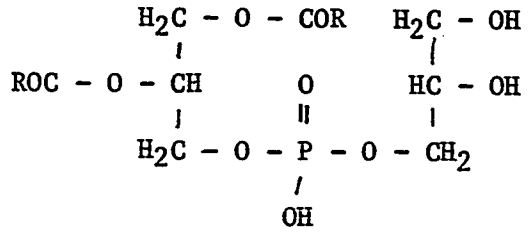
A. Monophosphatidylglycerols

Monophosphatidylglycerol is a compound composed of two glycerol units and a phosphate group which is esterified to both glycerols. The fatty acids are esterified either to both glycerols or only to one of them. There are several variations of this compound, found in nature, depending on position of the fatty acids on the glycerols, their number in the molecule as well as the position of the phosphate group between the two glycerols.

One of the main compounds of this type is the phosphatidylglycerol discovered by Benson and Maruo in 1958 (24) in alcohol extracts of Scenenedesmus cells. It was shown to be the main phospholipid in this plant. It has also been found in other plants such as tobacco, sweet clover, barley (24), spinach leaves (113), a wide variety of plant leaves, leaf chloroplasts, and fresh and soft water algae (26, 137, 149, 188, 271). This compound has also been found in numerous bacteria such as Bacillus cereus, Micrococcus halodenitrificans, Halobacterium cutirubrum, Micrococcus lysodeikticus, Staphylococcus aureus, Clostridium welchii and others (134, 155, 156, 173, 174, 232). In mammalian tissues, phosphatidylglycerol is a minor component and has only been detected in mitochondria (98, 248).

The concentration of the lipid is greatest in bacterial membranes, chloroplasts of plants and in mitochondria of animal cells. This characteristic distribution implies a special role of these compounds in the function of membranous structures of the living cells.

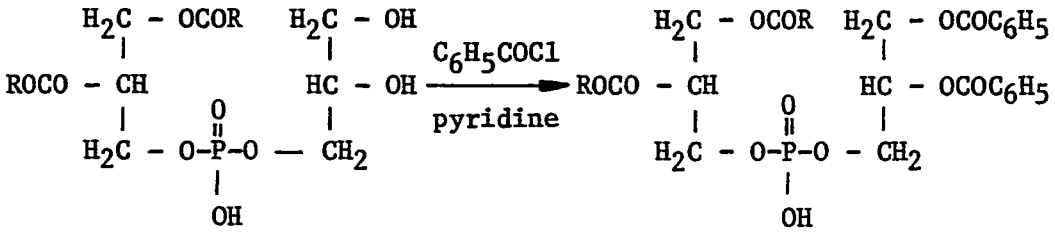
The chemical structure of phosphatidylglycerol was proposed by Benson and Maruo (24) and represents a molecule composed of two glycerol moieties, one phosphate and two fatty acids esterified to one glycerol in the following way:



This proposal is based on chromatographic and chemical analysis. The total lipids, extracted with alcohol, were separated by two-

dimensional paper chromatography into four distinct components. The individual lipids were eluted with pyridine and deacylated with o.2 N KOH, giving water-soluble products which could be identified chromatographically, electrophoretically and by analysis of the constituents. One of the products consumed two moles of periodate, which corresponded to two glycol units per mole of phosphorus in the molecule. This observation indicated an α,α'-diglyceryl-phosphate ester structure. Another analytical procedure, performed on the same compound, was oxidation with lead tetraacetate and subsequent hydrolysis of the oxidized product. The final product after this treatment was an ester (presumably glycerylphosphorylglyceraldehyde) which was chromatographically different from phosphatidylglycerol. These reactions are represented in Fig. 2.

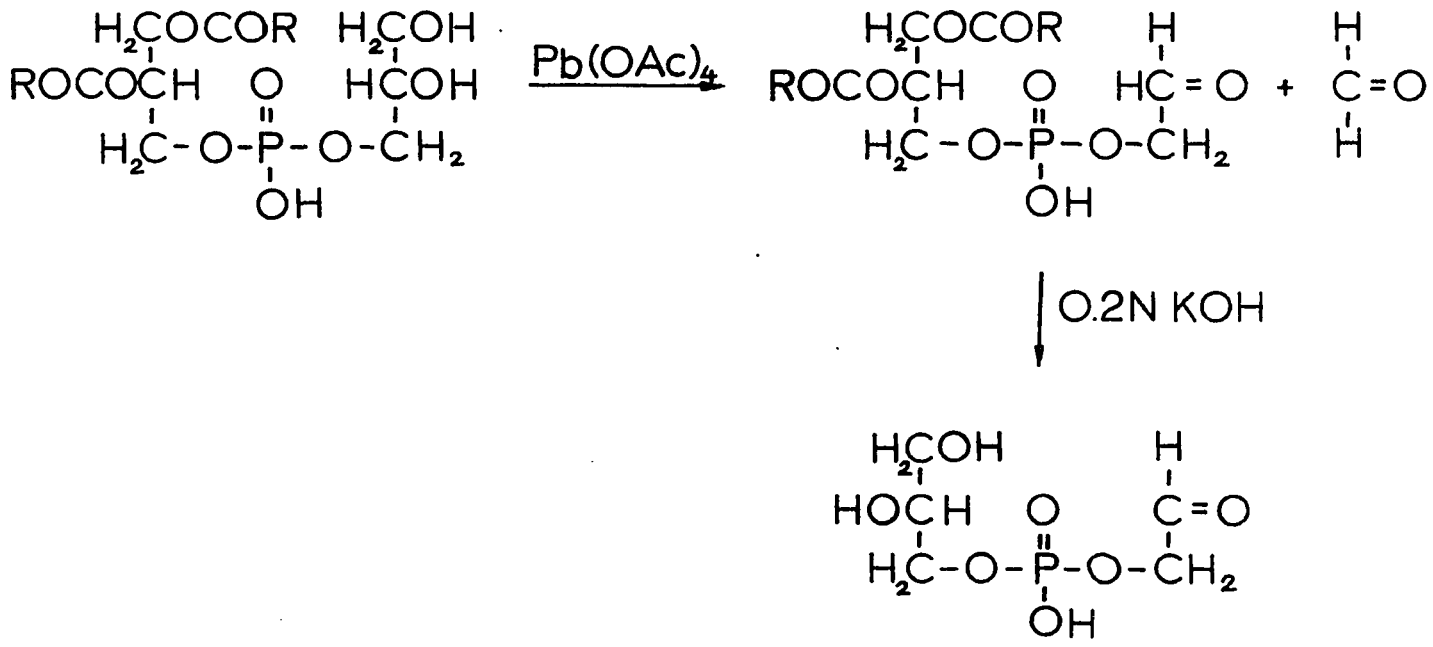
More support for the proposed structure was obtained by benzoylation with benzoyl chloride in pyridine which gave after deacylation a benzoate differing from the original lipid and the glycerylphosphorylglycerol. The benzoylation proceeded in the following way:



The fourth procedure was the preparation of an acetone derivative of phosphatidylglycerol, which on deacylation gave a new compound, a monoisopropylidene α,α'-diglycerylphosphate, which again gave a glycerylphosphorylglycerol on brief treatment with Dowex-50 H^+

F I G U R E 2

Oxidation of phosphatidylglycerol with lead tetraacetate [$Pb(OAc)_4$]


Pb(OAc)4
+
0.2N KOH

at 60°C (Fig. 3).

The results of these chemical reactions supported the proposed structure of this lipid, although the distribution and proportion of fatty acids was not examined. This investigation was done by Macfarlane who isolated phosphatidylglycerol in larger amounts from Micrococcus lysodeikticus (173) by silicic acid column chromatography and purification of the sodium salt by precipitation in ether at 2°C. The molar ratio of fatty acid ester to phosphorus was found to be 1.95:1. By oxidation of the intact lipid with periodic acid, one mole of periodate per mole of phosphorus was consumed with the production of one mole of formaldehyde. The water-soluble deacylated product consumed, however, two moles of periodic acid per mole of phosphorus, which agreed excellently with the proposed structure.

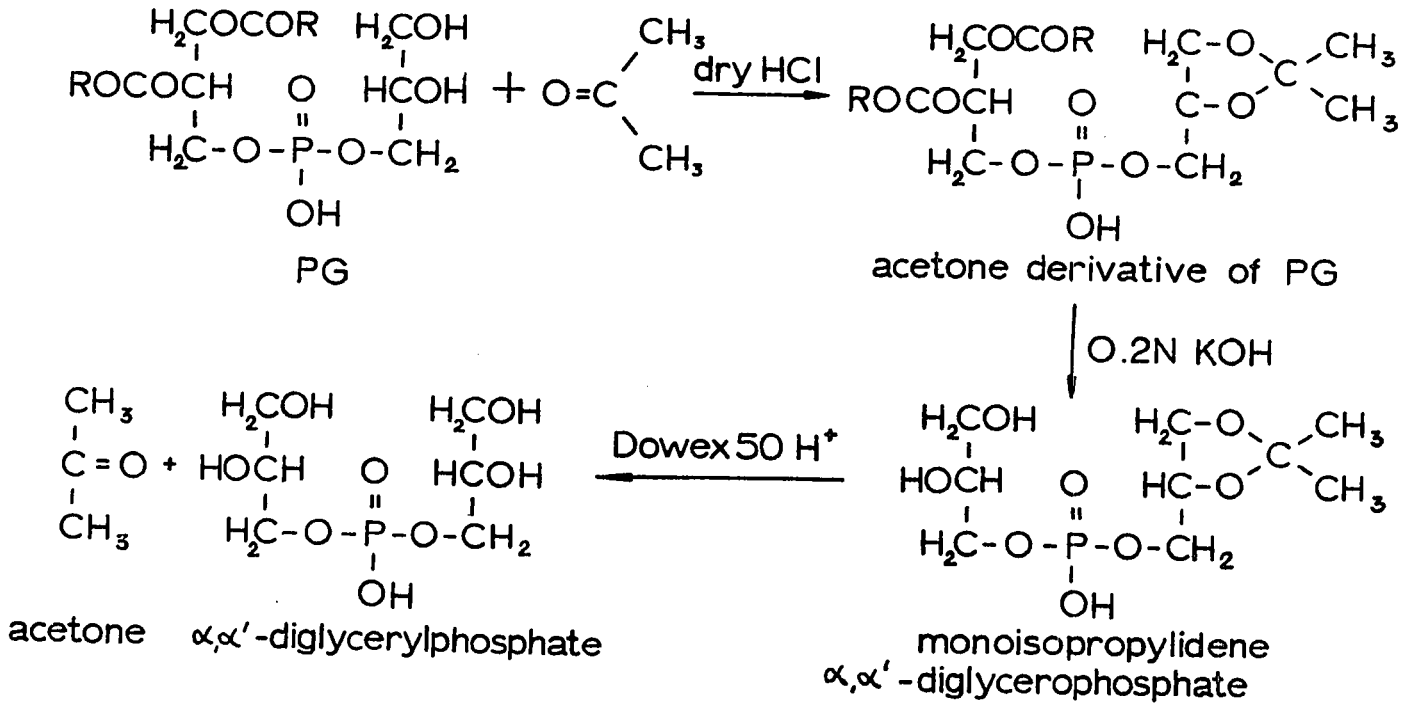
The final proof of the proposed structure came from the work of Haverkate, Houtsmuller and van Deenen (115) who confirmed its chemical structure by enzymatic hydrolysis.

In their experiments, phosphatidylglycerol was isolated from Bacillus cereus and Bacillus megaterium cultured in the presence of radioactive phosphate. The isolation procedure was essentially a fractionation on a silicic acid column, from which phosphatidylglycerol was recovered in the chloroform-methanol (94:6 v/v) effluent. It was further subjected to hydrolysis by phospholipases A, B, C and D, which specifically act on different bonds in the molecule according to the scheme in Fig. 4.

There is considerable confusion in the literature about the use of the specific letters A, B, C and D for these specific enzymes as

F I G U R E 3

Treatment of phosphatidylglycerol with acetone


PG
dry HCl
acetone derivative of PG
0.2N KOH
Dowex 50 H+
acetone
α,α'-diglycerylphosphate
monoisopropylidene
α,α'-diglycerophosphate

F I G U R E 4

Action of phospholipases on a phospholipid molecule

The specific attack of different phospholipases is shown.

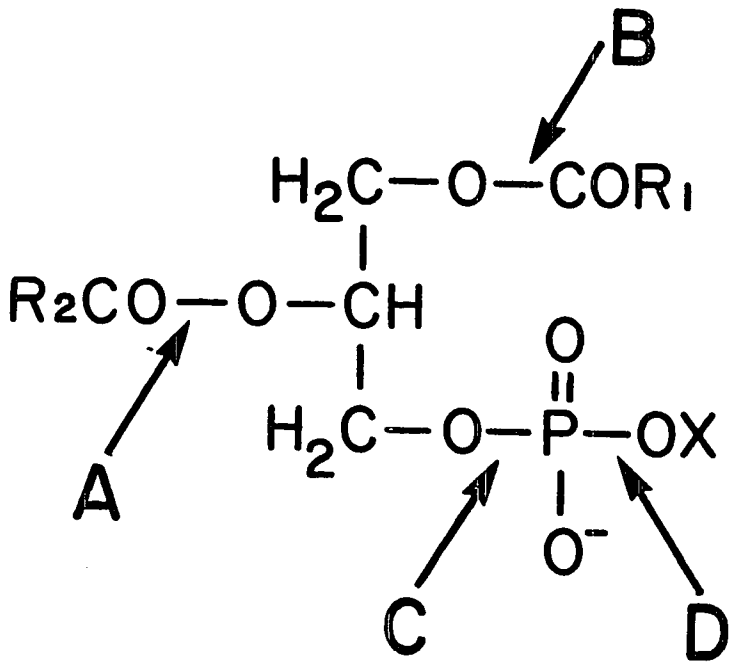
A - phospholipase A

B - phospholipase B

C - phospholipase C

D - phospholipase D

X - choline, ethanolamine, glycerol, phosphatidylglycerol, etc.


B
H2C—O—COR1
R2CO—O—CH
O
H2C—O—P—OX
O⁻
A
C
D

well as about the names of the enzymes involved. A variety of names was applied such as lecithinases, lecithases, lecitholipases, phosphatidolipases, phospholipidases, phcspholipases and phosphatidases. Kates (152) suggested the term phosphatidase, whereas Ansell and Hawthorne (9) preferred to use phospholipase.

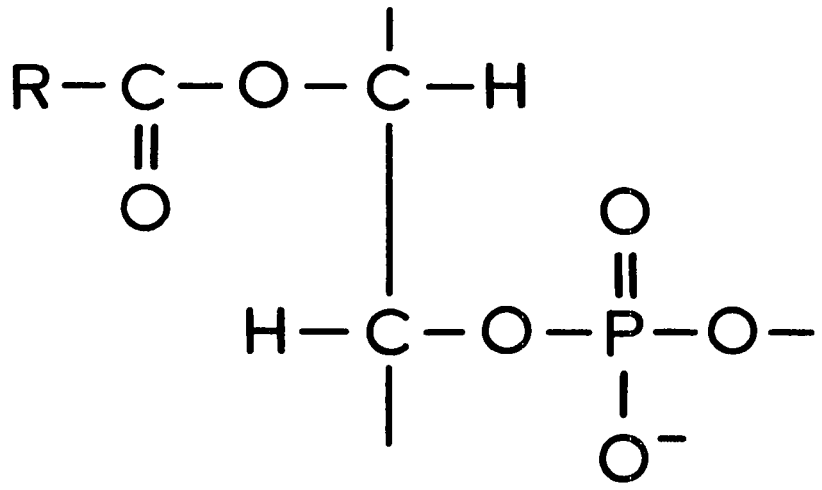
As seen on the scheme, phospholipase A is expected to hydrolyze the β-fatty acyl ester bond, which results in the liberation of a free fatty acid and a "lyso" derivative of the attacked phospholipid molecule. This enzyme has been found in snake, bee and wasp venoms, pancreas, kidney, bacteria and plants. Extensive study was done by van Deenen and his associates to establish its specificity. In the excellent investigation on the substrate specificity of phospholipase A by van Deenen and de Haas (78), it was established that the enzyme isolated from snake venom (Crotalus adamanteus) was highly specific for the β-fatty acyl ester bond. These investigations were performed with various synthetic substrates, where the positions of individual fatty acids were known. Some minimum structural requirements were established for these compounds to serve as substrates with phospholipase A. These requirements are illustrated in Fig. 5 and are composed of three main factors. The fatty acid attached must be in the β-position on the glycerol moiety. It was also shown that the blocking of the free hydroxyl function of the phosphoryl moiety caused inactivation of the substrate properties. Therefore, it was concluded that a free hydroxyl function on the phosphoryl moiety must be present. The third requirement is the oxygen-alkyl function, connected to the β-carbon atom. There is also another factor which

F I G U R E 5

Minimum structural requirements of synthetic phospholipids to serve as substrates for phospholipase A

The structural requirements as presented by van Deenen and de Haas (78) are illustrated.

Minimum structural requirements of synthetic phospholipids to serve as substrates for phospholipase A.



1. A free hydroxyl group on the phosphoryl moiety.
2. The β-fatty acid ester linkage.
3. The oxygen-alkyl function connected to the γ-carbon atom.

is very important and which is envisaged in the steric configuration of the molecule, favourable for interaction with phospholipase A. The enzyme is observed to be stereospecific and if we assume that the three indicated structural elements combine with three loci of the assymetric active site of phospholipase A, then only compounds which have the mentioned requirements will be in a favourable position for hydrolysis.

After action of pholpholipase B, a lyso compound is expected to result from removal of the fatty acid from the α-position. Phospholipase C would give a diglyceride, not soluble in water, and a glycerophosphate which can be recovered in the water phase whereas phospholipase D gives rise to phosphatidic acid and a free glycerol.

In the experiments with phosphatidylglycerol (115), the presence of free fatty acids could be demonstrated after treatment of this compound with phospholipase A and phospholipase B. It was also shown that the action of phospholipase B on the hydrolysate of phospholipase A gave a deacylated derivative identified as glycerylphosphorylglycerol, as expected.

Phospholipase C catalyzed a breakdown of phosphatidylglycerol in a Tris buffered medium (pH 7). A radioactive product, which came from P^{32} added to the medium, was obtained in the water phase, and diglycerides could be confirmed by TLC.

Phospholipase D split phosphatidylglycerol in an aqueous-ethereal system into phosphatidic acid and free glycerol, which could be demonstrated in the water phase.

This elegant procedure showed that phosphatidylglycerol was

susceptible under certain conditions to the action of the mentioned phospholipases, and it confirmed in a completely independent way the structure of phosphatidylglycerol first proposed by Benson and Maruo and further investigated by Macfarlane.

The chemical synthesis of this compound was performed by Baer (15) and Baer and Buchnea (16), who also established that the natural glycerophosphatides have predominantly the alpha structure and L-configuration.

The dioleyl-L, L-compound was synthesized by treatment of D-α,β-diolein with $POCl_3$ in pyridine. The product reacted with D-acetone glycerol to give dioleoyl-L-α-glycerylphosphoryl-L,α-glycerol. The corresponding distearoyl derivative was prepared by catalytic reduction of the unsaturated lipid. The whole procedure is represented in Fig. 6.

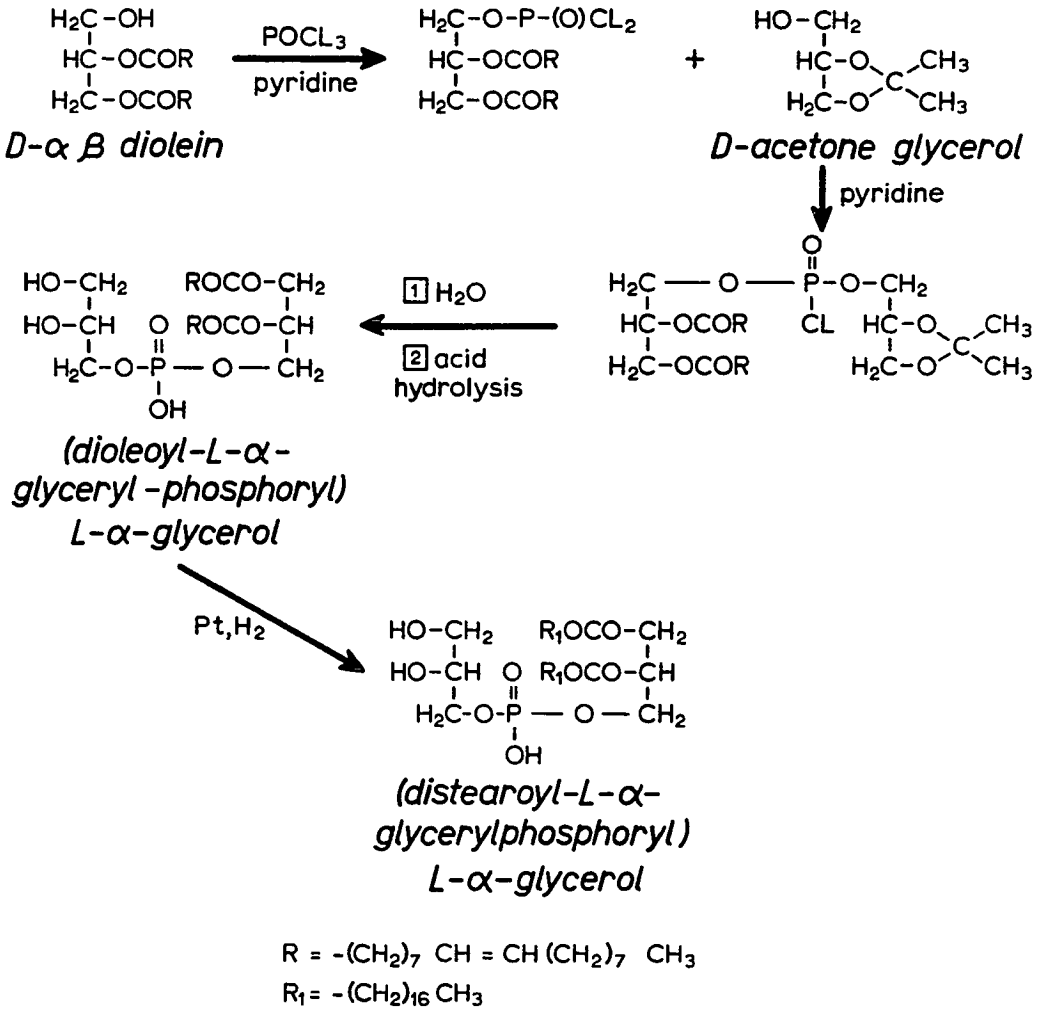
Other diglycerol phosphatides which differ in structure from phosphatidylglycerol, mentioned above, have also been isolated. Debuch and Rotsch (77) have isolated a new polyglycerophosphatide from spinach leaves with two moles of glycerol per mole of phosphorus but this compound could not be oxidized with periodate before deacylation. The alkaline deacylation product was also not identical with glycerylphosphorylglycerol. The proposed structure, which fits the data obtained, is a 3'-phosphatidyl-2'-glycerol compound which was also found not to be produced by a rearrangement of a phosphatidylglycerol molecule. The following structure was proposed:

F I G U R E 6

Chemical synthesis of phosphatidylglycerol

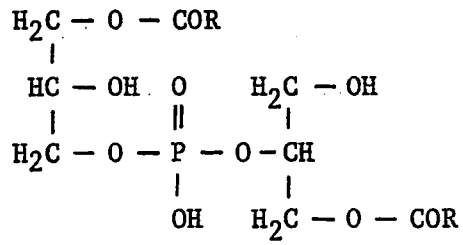
Synthesis of (dioleoyl-L-α-glycerylphosphoryl)-L-α-glycerol and (distearoyl-L-α-glycerylphosphoryl)-L-α-glycerol is presented (ref. 15, 16).

SYNTHESIS OF PHOSPHATIDYLGLYCEROL

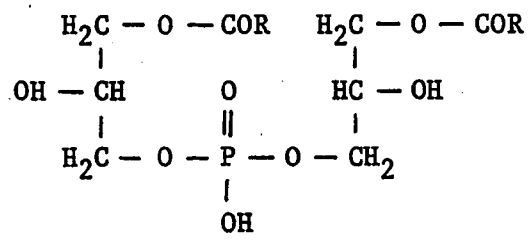


R = $-(CH_2)_7\ CH = CH\ (CH_2)_7\ CH_3$

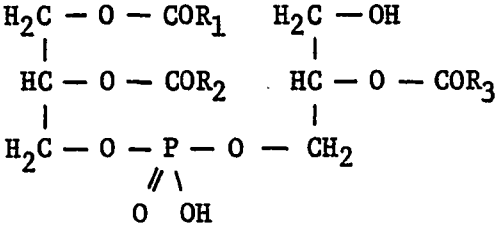
$R_1 = -(CH_2)_{16}\ CH_3$



Another phosphatidylglycerol isomer was isolated by Body and Gray (33) from pig lung and was reported also to be present in rat and rabbit lung. This lipid, which was found in the amount of 1% of total lipid phosphorus, also did not react with periodic acid but gave on deacylation glycerylphosphorylglycerol. The structure proposed is a lyso-bis-phosphatidic acid according to the following formula:



The same group also isolated another isomer from rabbit lung (34) with one more fatty acid per molecule. The compound was a semi-lyso-bis-phosphatidic acid and the following structure was proposed:



Wells and Dittmer (267) reported the isolation of a phosphatidylglycerol-type lipid from mammalian brain lipids. This lipid was shown to have one more phosphate group than phosphatidylglycerol and represented a phosphatidylglycerophosphate. The

deacylated product from this compound was isolated by Brundish, Shaw and Baddiley (44) from Pneumococcus. However, these authors concluded that this product probably arose from degradation of diphosphatidylglycerol (cardiolipin) which was found to be the major phospholipid in this organism. They also pointed out the possibility of degradation and intramolecular rearrangement of the glycerophosphatides. Phosphate could migrate from the end carbon to the central carbon of glycerol on storage of cardiolipin and its deacylated products in chloroform. They also obtained some evidence for intermolecular transesterification between the glycerylphosphorylglycerol molecules. In this way these compounds could arise from phosphatidylglycerol due to the mentioned changes or even by the degradation of diphosphatidylglycerol during its storage and isolation.

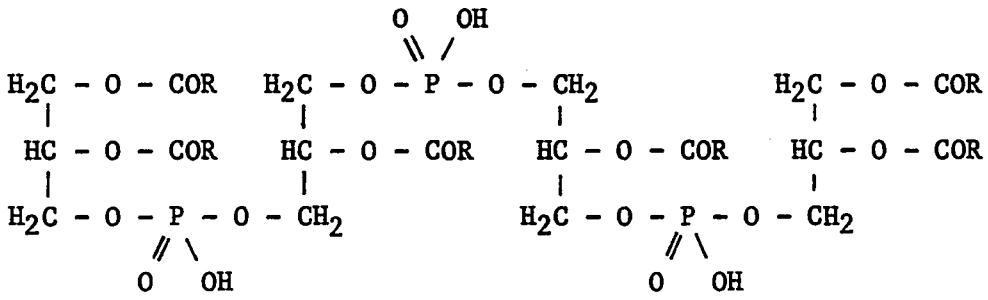
B. Diphosphatidylglycerol (cardiolipin)

The first isolation of diphosphatidylglycerol was performed by Pangborn, 1942 (205), from beef heart muscle. The new lipid was shown to be essential for the reactivity of beef heart antigen in the serologic test for syphylis, and the name "cardiolipin" was proposed. The substance was extracted with alcohol, precipitated with cadmium chloride and finally purified by fractionation in petroleum-ether and alcohol. The serologic activity could be demonstrated with this purified material as well.

In 1947, Pangborn (204) was able to propose a chemical structure for this compound, based on the following observations:

The ease of hydrolysis of cardiolipin with KOH served as the

basis for the degradation and study of the products obtained. On hydrolysis it yielded free fatty acids, which were recovered by petroleum-ether extraction and shown to be linoleic and oleic acids. The other product was a polyester of glycerophosphoric acid which was water-soluble and was not extracted with petroleum-ether. It was further precipitated with barium and the barium salt analyzed for barium, carbon, phosphorus and glycerol. The results agreed with the ones calculated from a formula on which four glycerols were connected with three phosphate groups, the fatty acids being esterified to the remaining hydroxyl groups of the terminal glycerols, as shown on the formula below:



It was soon recognized, however, that the molecule was composed of three glycerols and two phosphate groups. The first evidence for such structure came from McKibbin and Taylor (192) who isolated a polyglycerol phosphatide from dog liver. Using solvent and chromatographic fractionation procedures, they obtained a purified compound and analyzed it further. It contained no nitrogen and the glycerol, phosphorus and fatty acids were in a molar ratio of 3:2:3, which disagreed with the original formula proposed by Pangborn.

In 1956, Faure and Morelec-Coulon (85) modified the technique for the preparation of cardiolipin from beef heart. The heart muscle,

previously homogenized and freeze-dried, was extracted with methanol at room temperature. By a procedure of successive precipitation with barium and extraction with different solvents, the following substances were eliminated: non-phosphorylated lipids, lecithin, non-lipidic substances, phosphatides which contain nitrogen or inositol and finally the coloured impurities. Phosphatidylinositol, which was not serologically active and which accompanied cardiolipin in previous preparations, could be eliminated by insolubility of its acid form in ethanol.

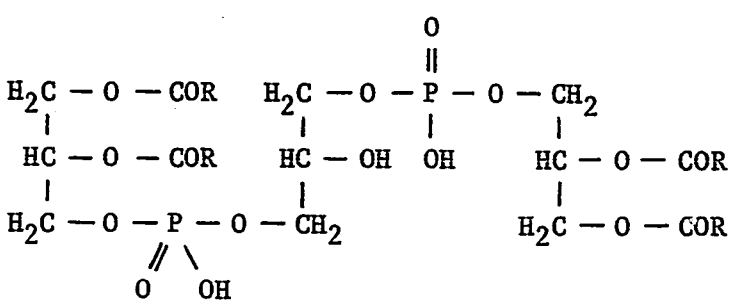
Due to the highly acidic character of cardiolipin, which can form salts in hydrochloric acid medium, it was possible to precipitate cardiolipin with barium chloride in an ethanolic solution. The barium salt, after repeated washings with ether and methanol and ether and acetone, could be completely separated from the nitrogen containing phosphatides which are soluble in the mentioned solvents. After elimination of barium with hydrochloric acid, the cardiolipin acid was converted with sodium hydroxide into its sodium form. The total yield was 3-4 g of cardiolipin from 1 kg of dry heart, which corresponds to 5 kg of the fresh heart muscle. Analysis of the isolated cardiolipin gave a ratio of glycerol:phosphorus:fatty acids of 3:2:4.

This result was further confirmed by Macfarlane and Gray (177) who obtained a cardiolipin from ox heart muscle by chromatography on silicic acid and by the methods of Pangborn and of Faure and Morelec-Coulon. A structure was suggested in which the molecule contains two phosphoric acids linking three glycerol residues with alcoholic

groups being esterified with fatty acids. There was one free alcoholic group in the middle glycerol.

Other evidence which was not compatible with the Pangborn structure came from Coulon-Morelec and Faure (66) and Macfarlane and Wheeldon (178). On heating with anhydrous acetic acid, cardiolipin was degraded to diglyceride without liberating any free fatty acid. The other main product was glyceroldiphosphate which arose from the splitting of a phosphoric acid diester bond. These bonds rupture preferentially under these conditions without rupture of fatty acid ester bonds. The glyceroldiphosphate and diglycerides were recovered in a molar ratio of 1:2, which is also incompatible with the Pangborn structure. Excluding the possibility of acyl migration during these degradations, the results clearly show that the fatty acids are localized on the terminal glycerols and that a free hydroxyl group exists on the glycerol which is located in the middle of the molecule.

Evidence was also obtained by periodate oxidation of the deacylated phosphoric ester which liberated one mole of formaldehyde per mole of phosphorus (176). The product had about one aldehyde function per phosphorus. All these results are compatible with the presence of a free hydroxyl group in the molecule and the following structure was finally proposed in 1957 by Macfarlane and Gray:



The first direct evidence, however, for a free hydroxyl group came from Faure and Coulon-Morelec (83) who, on examining the serologic activity of cardiolipin, reported an esterification of the free hydroxyl group of cardiolipin with acetic or oleic acid. The resulting compound still retained some of the original serologic activity.

Another interesting approach to the elucidation of the structure of cardiolipin by chemical degradation and transformation of the molecule came from LeCocq and Ballou (163). They investigated the structure of beef heart cardiolipin and a diphosphatidylglycerol from Mycobacteria. The reactions which were used in these investigations allowed the selective removal of α-linked glycerol from the molecule, yielding glycerol 1,3-diphosphate. The compound isomeric to the proposed structure, where the linkage of one phosphate is on the 2-position of glycerol, would in this case yield glycerol 1,2-diphosphate, whereas the compound with the Pangborn structure yields diglyceroltriphosphate. The reactions involved are represented in Fig. 7.

The degradation product, glyceroldiphosphate, was indistinguishable chromatographically from glycerol-1,3-diphosphate synthesized to serve as a reference compound. No 1,2-isomer or diglyceroltriphosphate was obtained. These findings were consistant in both beef heart and Mycobacterium phlei cardiolipins.

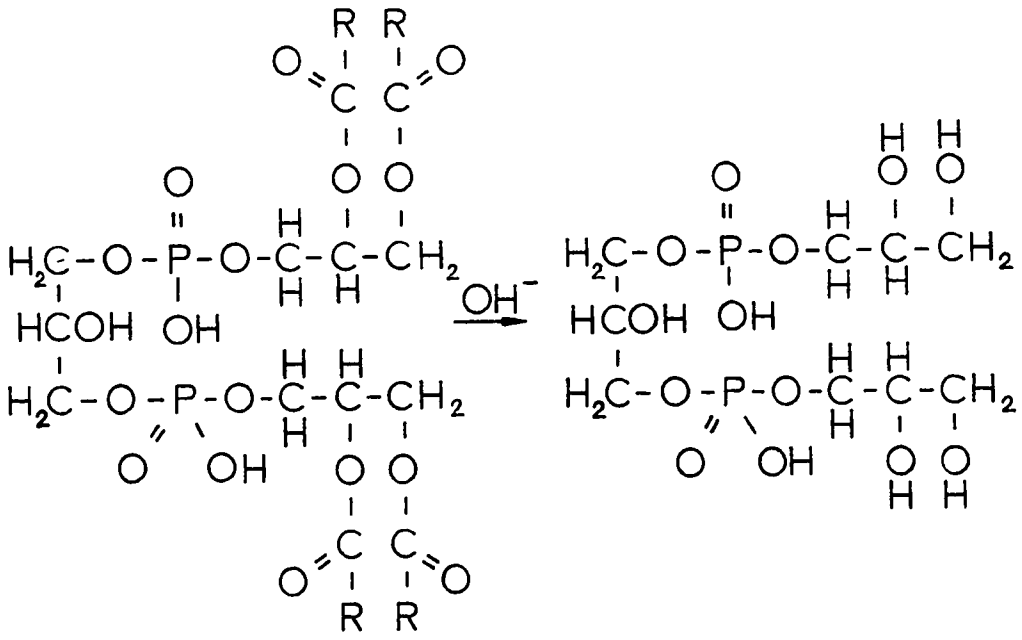
Much evidence for the diphosphatidylglycerol structure of cardiolipin had accumulated by 1964 and it seemed that it was generally accepted without necessity for further exploration. However, this

F I G U R E 7

Chemical treatment of diphosphatidylglycerol.

Selective removal of α-linked glycerol moieties.

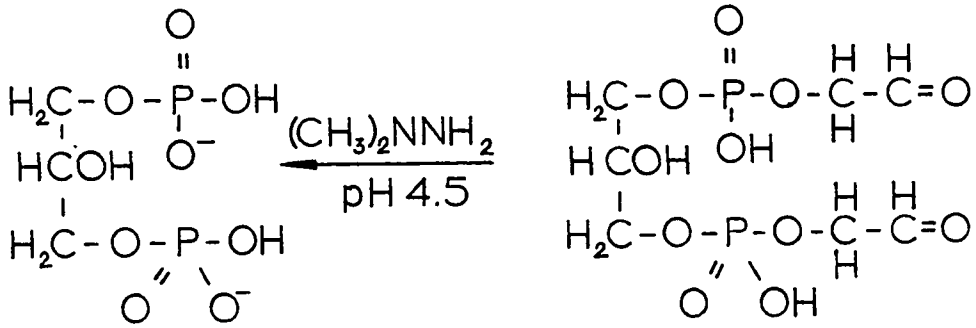
DPG - diphosphatidylglycerol



DPG

deacylated DPG

$NaIO_4$



glycerol-1,3-diphosphate

was not the case. Many investigators were unable to reproduce the results supporting the diphosphatidylglycerol structure, probably due to the difficulty of preserving the primary structure of the compound during isolation procedures.

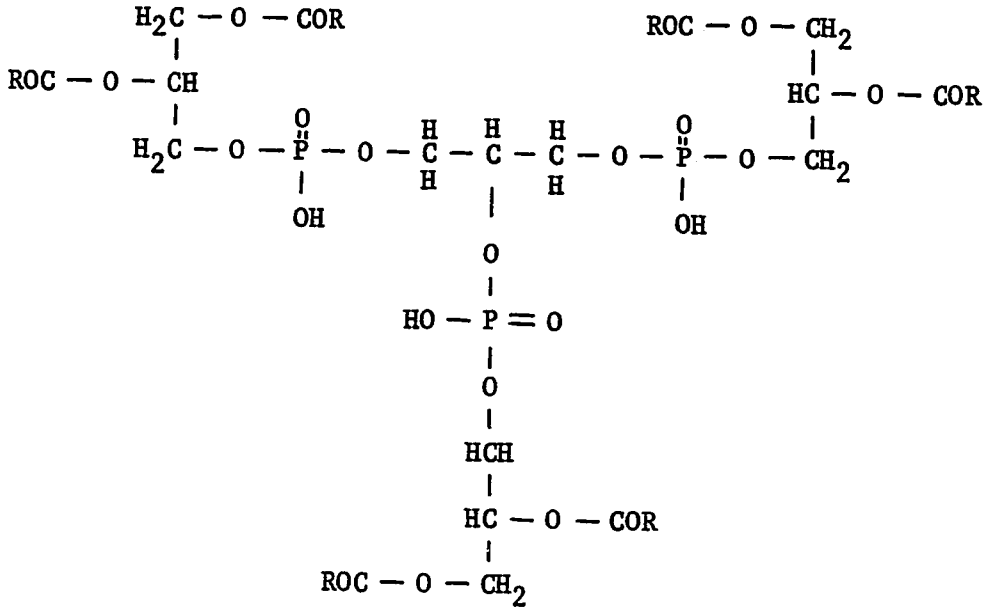
Rose (223) found that the diphosphatidylglycerol structure does not fit with the molecular structure of rat liver cardiolipin obtained by extraction with chloroform-methanol and chromatography on two successive silicic acid columns. The columns were eluted with chloroform-methanol and five fractions were collected which contained phosphorus but no phosphatidylethanolamine, phosphatidylcholine, phosphatidylinositol, sphingomyelin and neutral lipids.

Repeated attempts at acetonation and acetylation of the free hydroxyl group in the isolated cardiolipin were unsuccessful. It seems likely that there is considerable steric hindrance and the failure of the reaction is rather due to the lipid character of the molecule than to the non-reactivity of the -OH group. Glycerol diphosphate, which contained a free hydroxyl group on the glycerol moiety, was produced during acetonation. This compound, however, could be subsequently acetonated, which shows that there could be a steric reason (106) for the failure to acetonate the free hydroxyl group present in cardiolipin.

The other possibility proposed by Rose was that there was no free hydroxyl group in the molecule, but that a glycerophosphate was esterified through a phosphodiester to the hydroxyl of glyceroldiphosphate in the center of the molecule. If this were the case, the bond would be extremely reactive and would easily generate a free -OH

group under acidic conditions. In this case, one mole of glycerophosphate or phosphatidic acid would be liberated with one mole of glyceroldiphosphate in the course of acetic acid hydrolysis. The quantity of phosphatidic acid and glycerophosphate, liberated during the hydrolysis was studied by Macfarlane and Wheeldon (178) whose results agreed with this proposal.

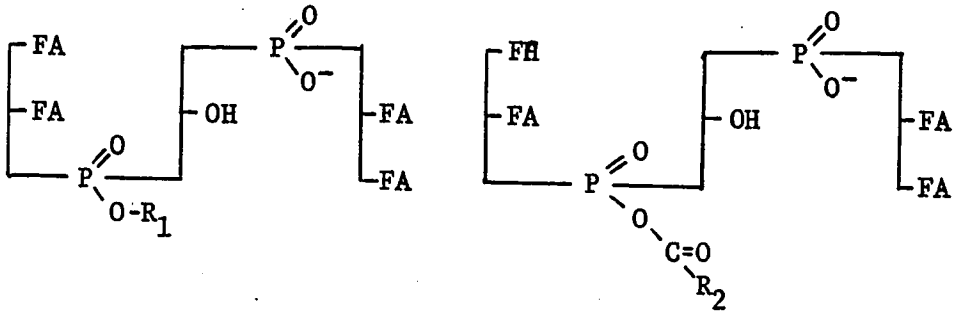
From these considerations, Rose proposed a structure of cardiolipin, which was in agreement with the diphosphatidylglycerol structure but represented rather an isomer of the originally proposed Pangborn structure. Cardiolipin in this case would consist of four glycerol units with three phosphate groups and six fatty acids, according to the following formula:



Despite the lack of a free -OH group, this compound would give glyceroldiphosphate on acetic acid hydrolysis as mentioned earlier.

Another interesting investigation in this field was performed by Courtade, Marinetti and Stotz (68) who analyzed cardiolipins from

different rat tissues. Whereas beef heart cardiolipin has a structure consistent with the one proposed by Macfarlane and Gray, rat tissue cardiolipins did not seem to have the same structure. They were also not as susceptible to acetic acid hydrolysis as beef heart cardiolipin. Since only diglycerides were produced by acetic acid hydrolysis, the Pangborn structure was excluded and a new proposition made in which there was an extra fatty acid which could be esterified on the one of the free -OH groups of two phosphates present in the molecule. Another difference from beef heart cardiolipin was the presence of vitamin A and another unidentified quinone bound to the phosphate group. According to these findings, two alternative structures (I and II) were presented, although the possibility of mixtures was not excluded:



I II

(R_1, vitamin A or other similar group; R_2, fatty acid side chain. FA, stands for fatty acid.)

The final proof of the ox heart cardiolipin structure came from the excellent study of this compound by de Haas, Bonsen and van Deenen (106). They were able to synthesize model compounds and to compare

their structural characteristics with the naturally occurring lipid. Beef heart cardiolipin was compared with synthetic polyglycerolphospholipids such as diphosphatidylglycerol, a long chain fatty acid ester of diphosphatidylglycerol, bis-phosphatidic acid and phosphatidylglycerophosphate. The formulae of these compounds are represented in Fig. 8.

The starting substance for the synthesis of diphosphatidylglycerol was a silver benzyl diacyl-L-α-glycerophosphate which was reacted with an α,β-di-iodoglycerol derivative which had the β-hydroxyl group protected by a tert-butyl ether function. After chromatography of the reaction product on silica, the bis-triphosphate ester was obtained in a yield of about 65%. Phosphate-protecting benzyl groups were removed by treatment with barium iodide in acetone and the tert-butyl ether function was split off with anhydrous hydrogen chloride. Diphosphatidylglycerol (DPG) was obtained in analytically and chromatographically pure form in a yield of 69% based on bis-triphosphate ester. The individual steps of this synthesis are represented in Fig. 9.

The following characteristics of cardiolipin and the synthesized compounds were investigated and compared: melting points, optical rotations, infrared spectra, purity of intermediates by paper and thin-layer chromatography, quantitative analysis of fatty acids by GLC and enzymatic degradation with phospholipases A, C and D.

Melting points and optical rotations of cardiolipin and DPG corresponded well, but no informative results could be obtained from infrared spectra. The synthesized diphosphatidylglycerol and natural

F I G U R E 8

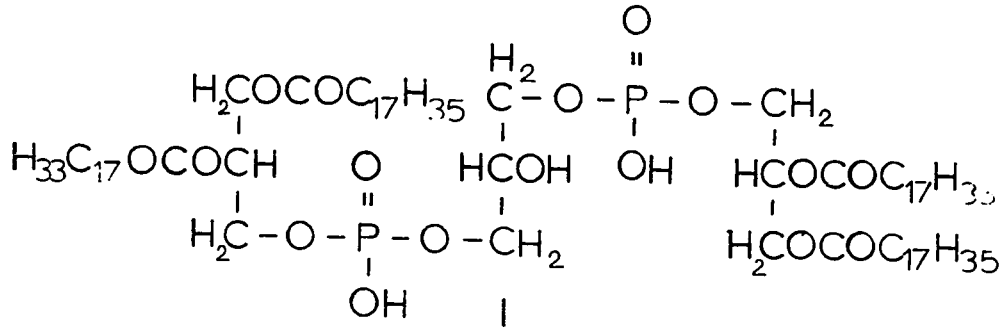
Synthetic substrates used for comparison with beef heart cardiolipin

I - diphosphatidylglycerol (DPG)

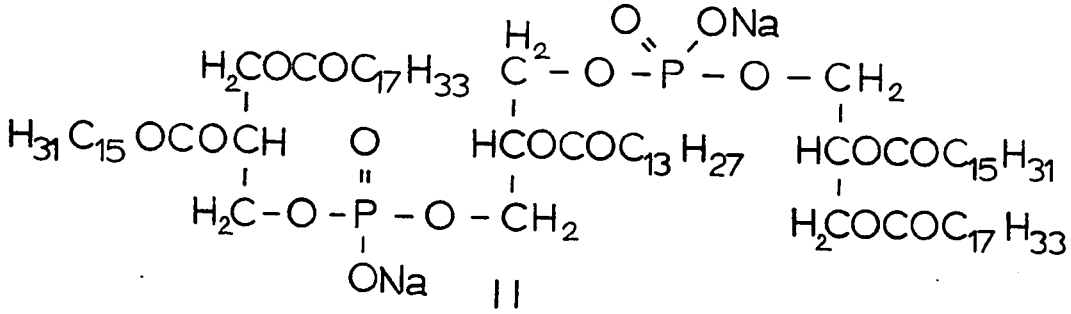
II - long chain fatty acid ester of DPG

III - bis-phosphatidic acid (phosphatidyl diglyceride)

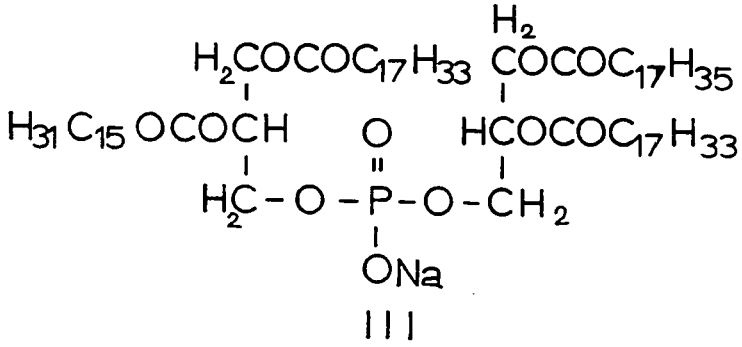
IV - phosphatidylglycerophosphate



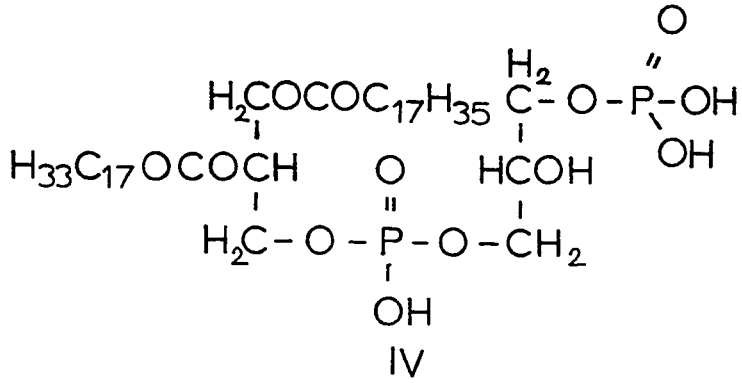
diphosphatidylglycerol



long chain fatty acid ester of DPG



bis phosphatidic acid



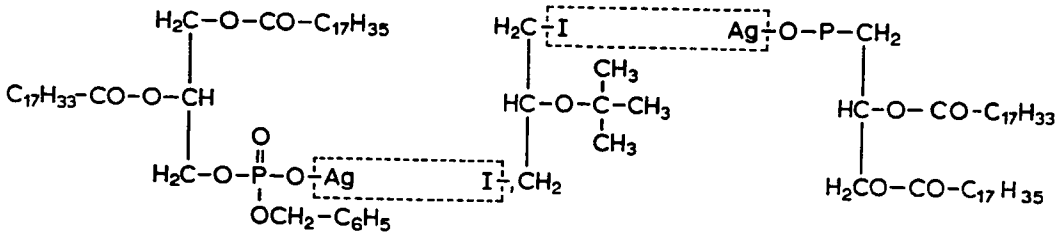
phosphatidylglycerophosphate

F I G U R E 9

Synthesis of diphosphatidylglycerol (DPG)

The individual steps during chemical synthesis of DPG are shown acc. to de Haas, Bonsen and van Deenen (106).

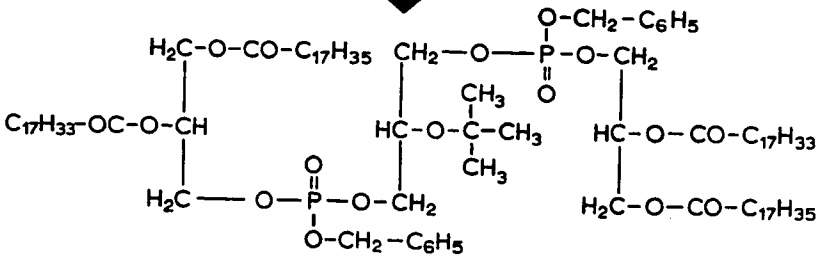
SYNTHESIS OF DIPHOSPHATIDYLGLYCEROL



Ag-benzyl diacyl-L-α-glyceryl phosphate
I

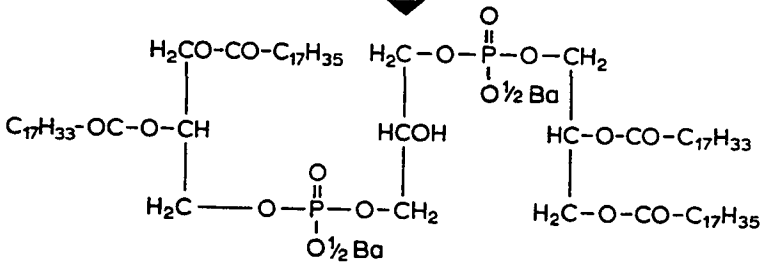
α,γ-di-iodoglyceryl β-t-butyl ether
II

I



Bis triphosphate ester
III

[1] Ba I_2 in acetone
[2] HCl



Diphosphatidylglycerol
IV

cardiolipin had identical R_f values on TLC, and their deacylated products could be shown by paper chromatography to be identical. Acylation of DPG as well as cardiolipin was not very successful and only little esterification was observed. As the authors concluded, the reason for this was probably steric hindrance and/or low reactivity of the secondary hydroxyl group.

Conclusive evidence for a diphosphatidylglycerol structure of cardiolipin came from study with phospholipases. The specificity of different phospholipases is mentioned earlier (Fig. 4).

Both synthetic DPG and cardiolipin were susceptible to the action of phospholipase A (crude lyophilized venom of Crotalus adamanteus) but the hydrolysis proceeded very slowly. Two lyso compounds were obtained in which one or two fatty acids were missing and which were chromatographically identical for both DPG and cardiolipin.

Degradation of DPG with phospholipase C (from Bacillus cereus) proceeded according to the scheme in Fig. 10. A diglyceride could be demonstrated in the ethereal phase after incubation of both compounds with this enzyme. Soluble glyceroldiphosphate was found in the aqueous phase. By comparing with the separately synthesized 1,2- and 1,3-glyceroldiphosphate, it appeared that the enzymatic degradation of DPG as well as ox heart cardiolipin gave rise to the 1,3- isomer only.

Phospholipase D from Brussels sprouts was unable to degrade synthetic diphosphatidylglycerol or cardiolipin. This enzyme was also not able to degrade the synthetic phosphatidylglycerophosphate, acyl

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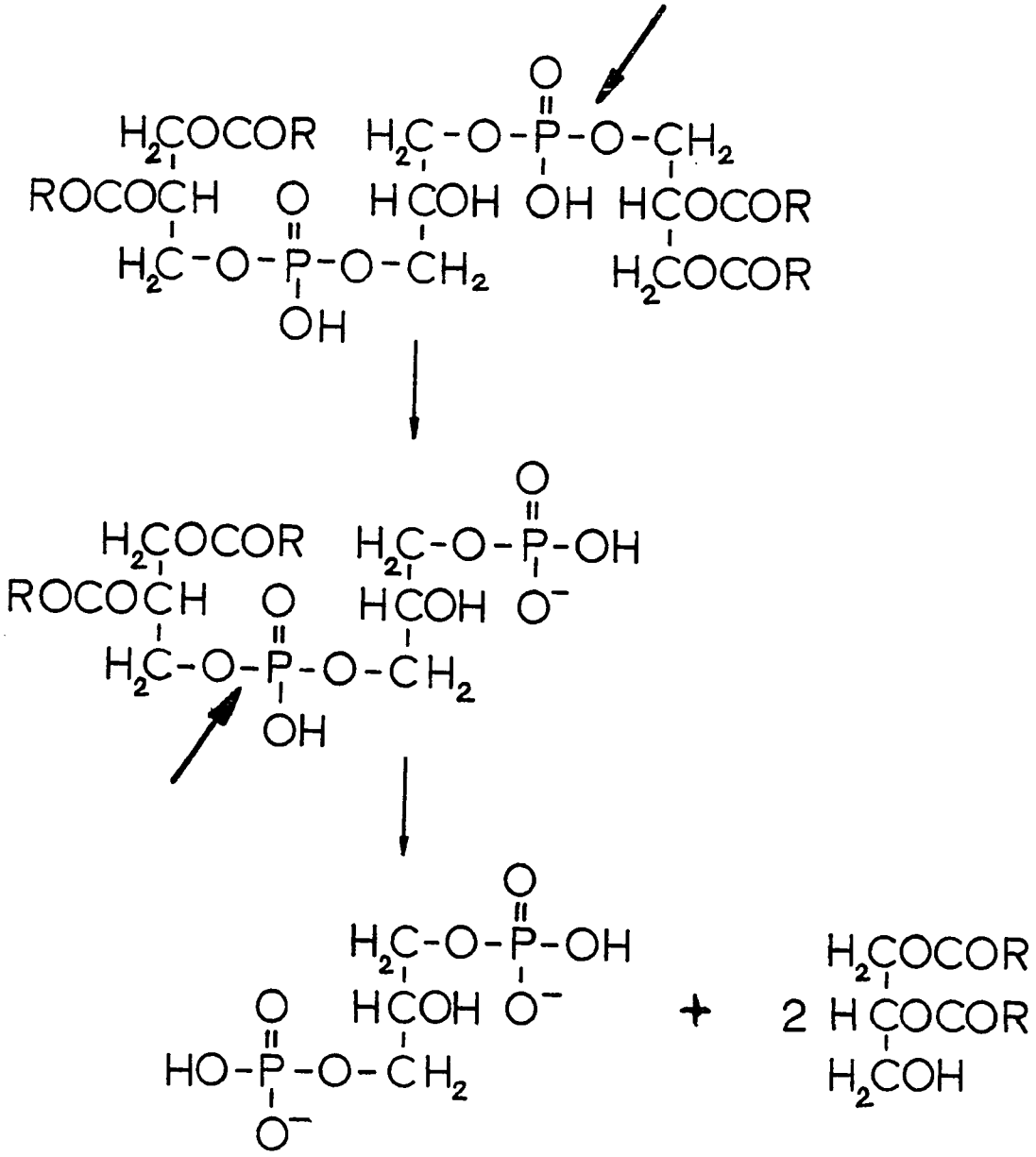
Action of phospholipase C on diphosphatidylglycerol

The arrows indicate the sites of attack of phospholipase C.



derivative of diphosphatidylglycerol and bis-phosphatidic acid.

Diphosphatidylglycerol was also isolated by Macfarlane from ox liver (170) and Getz and Bartley isolated the same compound from rat liver mitochondria (93). It was also found in fish and avian muscle(99).

Benson and Strickland isolated cardiolipin from plants and algae (25), and showed that its structure was in agreement with the one proposed by Macfarlane and Gray. Isolation of cardiolipin from yeast was performed by Kates and Baxter (154) and it was isolated from Mycobacteria by Faure and Marechal (84). The isolation of cardiolipin from different Gram-positive bacteria was done, among others, by Macfarlane (173, 174).

C. Fatty acid composition of phosphatidylglycerols

Pangborn (204) was able to identify the fatty acids of ox heart cardiolipin as linoleic and oleic acids in a proportion of 5 : 1. Other investigations of ox heart and liver, pig spleen, lung and kidney and rat liver cardiolipins, confirmed this distribution and one can say that the fatty acids in mammalian tissue cardiolipins are unsaturated with 60 - 80% linoleic acid and 10 - 20% oleic acid. Some other acids have also been found in different tissue samples. Ox heart and ox liver cardiolipins contain 8 - 15% linolenic acid and those from pig about 10% palmitic and stearic acids (100).

The "polyglycerophosphatide fraction" isolated by Chang and Sweeley (53) from dog adrenal glands contained 45% linoleic, 20% oleic, 17% palmitic and stearic and 9% polyenoic acids. However, this

preparation does not seem to be completely pure, so that these results should be interpreted accordingly.

Diphosphatidylglycerol from trout muscle (99) contained, however, mainly oleic acid (45%) and the other acids were palmitic (19%), stearic (14%) and linoleic acid (6%).

Biran and Bartley (29) investigated fatty acids from a polyglycerolphosphatide fraction of rat brain mitochondria and found 34% of saturated acids (palmitic and stearic), 19% oleic, 10% linoleic and 15% polyenoic acids.

Courtade, Marinetti and Stotz (68) found by analyzing rat tissue cardiolipins that the distribution of fatty acids was not uniform in all tissues investigated. The fatty acids in heart, kidney and liver were found to be highly unsaturated, whereas other tissues such as brain, lung and testes had highly saturated acids. The main fatty acid in heart, kidney and liver was linoleic (60 74%). In brain, stearic dominated (46.3%) followed by oleic (27.8%). Muscle and lung had about 40 - 43% stearic and lung compared to muscle had more saturated than unsaturated acids. Testis had predominantly saturated acids (72%), palmitic being the main acid present (55%).

The fatty acid distribution in polyphosphatidylglycerols varies considerably in different species of bacteria (175). In Micrococcus lysodeikticus the main fatty acids are saturated C_{15} branched chain acids with a high proportion of the anteiso, 12-methyltetradecanoic acid. In Staphylococcus aureus, there are about 70% of branched chain acids, mainly C_{15}, and 20% of normal saturated acids (C_{16}, C_{18}, C_{20}). Diphosphatidylglycerol from Mycobacterium tuberculosis contains equal

amounts of oleic and palmitic acids.

According to Gray (98), there is considerable difference in fatty acid composition between phosphatidylglycerol and diphosphatidylglycerol isolated from normal rat liver. On the other hand, phosphatidic acid, isolated by Hubscher and Clark from mammalian liver (130) was very similar to cardiolipin in fatty acid composition.

The following fatty acids were found in phosphatidylglycerol from rat liver mitochondria (98): oleic (20.9%), linoleic (19.7%), stearic (14.1%), eicosadienoic (13.4%) and palmitic (12.0%). There were also minor quantities of some other acids. The distribution of fatty acids in phosphatidylglycerol from different plants varies and the data have been summarized by James and Nichols (137).

The positional distribution of fatty acids in phospholipids is usually studied by the use of phospholipase A obtained from snake venom (106, 180, 183, 200,201). It could be demonstrated that the distribution of fatty acids in cardiolipin from Mycobacteria was non-random (200). The shorter chain fatty acids (C_{16}) were mainly esterified in the β-position, whereas the α-position of the glycerols was preferentially occupied by longer chain fatty acids (C_{18}). Brockerhoff et al. (40) found the same distribution in triglycerides. The shorter and more unsaturated acids showed a greater tendency to occupy the β-position of glycerol, the longer and the more saturated ones, the α-position. The same findings could be demonstrated in beef and human cardiolipins (201), and in phosphatidylethanolamine and phosphatidylcholine of A. agilis, E. coli, A. tumefaciens, C. butyricum and S. marcescens (120). According to all these findings,

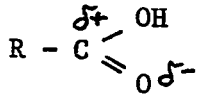
it seems that the fatty acid distribution in phospholipids is not random but rather specific, so that the shorter and more unsaturated fatty acids preferentially occupy the β-position, whereas the longer and less unsaturated acids tend to occupy the α-position of the corresponding glycerol.

III. CHEMISTRY OF CARBOXYLIC AND PHOSPHATE ESTERS

The phospholipids with which we are concerned in the present investigation are esters of glycerol with fatty acids and phosphoric acid. As esters have some common characteristics and the ester linkages have an influence on the behaviour and arrangements of phospholipid molecules, it would be of interest to include a short discussion about the chemistry of these compounds. Of special interest is the behaviour of esters under acidic and basic conditions, as most of the analytical procedures which are associated with the characterization of various phospholipids involve acid or base hydrolysis of the original compound.

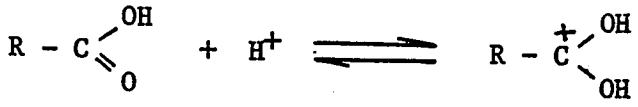
A. Carboxylic esters

Esterification and hydrolysis of esters are closely interrelated processes. A general formula of a carboxylic acid can be represented as follows:



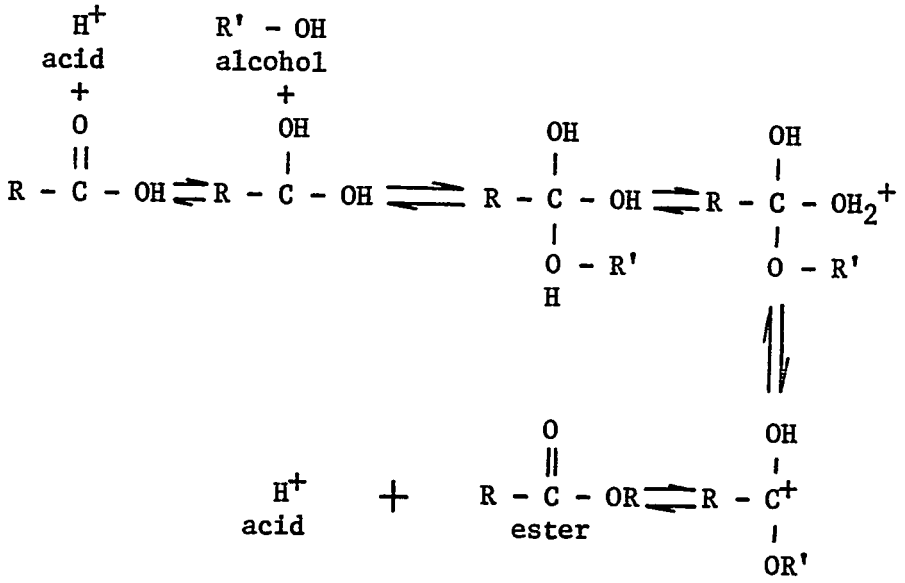
The -OH group is replaced in esterification but it is the

-C=O group which makes this replacement possible, as the π-electrons of the carbon-oxygen double bond are pulled strongly towards the more electronegative oxygen atom, creating in this way a dipole moment in the molecule. Due to this effect, the carbon atom is deficient in electrons and susceptible to nucleophilic attack by bases. Carbonyl oxygen, being rich in electrons, is at the same time susceptible to electrophilic attack by acids. In the presence of acid, H^+ becomes attached to the carbonyl oxygen. The effect of this is that the carbonyl carbon becomes even more electropositive, which results in a greater susceptibility to nucleophilic attack. This situation is represented by the following equilibria:



The basic alcohol molecule is the nucleophilic reagent in the esterification of acids, whereas during hydrolysis of esters, the nucleophilic reagent is the basic water molecule. One can summarize both processes in saying that there is a nucleophilic substitution of -OR' for -OH in esterification and -OH for -OR' in hydrolysis.

The common accepted mechanism (193) for both esterification and hydrolysis of esters is represented by the following equilibria:



There are also some other factors which should be considered in esterification and hydrolysis of esters. Both of these two processes are quite sensitive to steric hindrance so that they cannot take place if the reacting groups are in a sterically inconvenient position for nucleophilic attack. Also, when bulky groups are present in the molecule, whether in the alcohol or acid part, the formation of the ester is more difficult and such compounds are less stable. Hydrolysis in this case proceeds more easily and detachment of these groups from the molecule takes place.

B. Phosphate esters

Orthophosphoric acid is a "medium-strength" acid with three -OH groups which ionize with pK values at pH 2.0, 7.0 and 12.4. Since the middle value coincides with neutrality, salts of orthophosphoric acid can form an ideal physiological buffer system. Normally the strongly acid group is permanently esterified and so in the form of a buffer it exists as mono-or dibasic salt which are in equilibrium ($NaH_2PO_4 \rightleftharpoons Na_2HPO_4$).

Glycerol, on the other hand, can act as a polyacidic base which can be esterified either with different fatty acids to form mono-, di- and triglycerides or with phosphoric acid to form glycerophosphates. The phospholipids are then in most cases compounds where the fatty acids are esterified to the 1- and/or 2-position of the glycerol and the position 3- is esterified by a phosphate group. The phosphate usually carries other groups such as choline, ethanolamine, glycerol, etc.

In orthophosphoric acid, which is the acid predominantly involved in phospholipid structures, the phosphorus is pentavalent and forms three covalent bonds, sharing its electrons with the oxygen of the hydroxyl group. Each pair of electrons represents in this way a covalent bond. The other double bond, P=O, is formed with its own pair of electrons. Because of the high electronegativity of the oxygen in the molecule, the electrons concentrate on the phosphate group and a dipole is created between this group and the adjacent carbon from the glycerol. In this way the whole molecule is electrostatically polarized and has a hydrophilic part associated with the phosphate ester and a hydrophobic part associated with the long hydrocarbon chain of the fatty acids. The whole molecule is probably, because of this effect, specifically oriented in the cell membrane as a function of orientation of other cellular constituents, such as proteins.

A further characteristic of the orthophosphate is its strongly resonant nature which allows any of the four oxygen atoms to become the double bonded one. This has the effect of shortening the bonds

between the groups which can then approach more closely to one another and interact more easily (197). An important effect which phosphoric acid can have in the molecule is to increase the acidity of other acid groups with which it is associated. There is also a reciprocal effect which is manifested in the effect of organic hydroxy compounds, such as hexoses, on the phosphate hydroxyl groups. They can increase the acidity of the latter groups so that the ionization of the three phosphate hydroxyl groups can be shifted about one pH unit compared with free orthophosphoric acid (197).

There are three types of esters possible with orthophosphoric acid: mono-, di- and tri-esters. Generally all these compounds are relatively stable (41). Under acidic conditions, the mono- and diesters are more stable than the triesters. Diesters and monoesters are also very resistant to base hydrolysis whereas the triesters are hydrolyzed more readily. A trimethylphosphate, for example, undergoes hydrolysis in alkali which proceeds by P-O fission to give a dimethylphosphate which is then extremely resistant to further hydrolysis.

The rate of hydrolysis of organic phosphate esters was studied by Plummer and Burch (212), who examined the rate of acid and alkaline hydrolysis of different di- and tri-phosphate esters. For example, they have treated diethyl- and triethyl-phosphate with 2N NaOH at 90°C and 2 N H_2SO_4 at 90°C also and found the following: In the case of alkaline hydrolysis, 0.92 moles of alcohol were removed from the triethyl-phosphate after 30 hours of treatment. No hydrolysis was observed with diethyl-phosphate even after 96 hours. In the case of

acid hydrolysis, 0.84 moles of alcohol were removed from triethyl-phosphate after 8 hours of treatment and 2.5 moles after 360 hours. Diethyl-phosphate liberated under the same conditions, after 20 hours, 0.045 moles of alcohol and after 386 hours, 1.87 moles of alcohol. These results show that the phosphate diesters and triesters can be completely hydrolyzed with sulphuric acid, whereas the alkali cannot hydrolyze the diethyl-phosphate. However, under the same conditions, all the possible esters with phenol could be completely hydrolyzed with alkali. (Diphenyl: 1.01 mole phenol recovered after 76 hours of acid hydrolysis at 85° and 1.5 moles after 70 hours alkaline hydrolysis; triphenyl: 1.95 moles of phenol recovered after 144 hours acid hydrolysis and 3.0 moles recovered after 170 hours alkaline hydrolysis.)

The mechanism of hydrolysis of organic esters of phosphoric acid has been discussed by Barnard et al. (20), Henbert and Lovell (119), Hudson and Harper (132), Morrison and Boyd (193), Brown (41), Brewster and McEwen (38), Samuel and Silver (230) and Cherbuliez and Leber (57, 58).

There are four molecular species in a monosubstituted ester of phosphoric acid (20) which differ in degree of protonation and are represented by the following formulae:

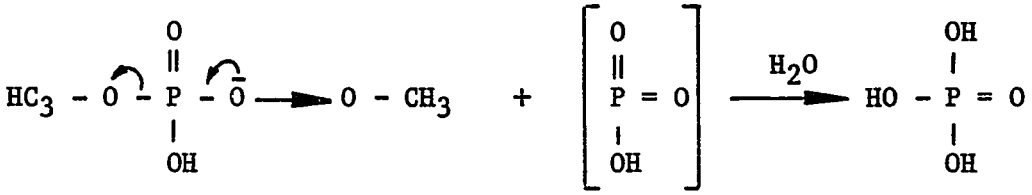
(a)

(b)

(c) (d)

In the di- and tri-substituted esters, there are three and two species respectively. Hydrolysis of such a compound may proceed by a unimolecular or bimolecular process. It results from nucleophilic attack on either the carbon atom or the phosphorus atom of the ester linkage, due to their electropositive dipole character. It was shown by Barnard et al. (20) that water hydrolysis of methyl phosphate follows strictly first order kinetics. The mechanism is discussed for the species (b) and two possibilities are suggested. The first is a bimolecular attack on the phosphorus atom by a solvent molecule as follows:

The other possibility is a slow heterolysis of the phosphorus-oxygen bond to give as a result a hypothetical meta-phosphoric acid which rapidly gives orthophosphoric acid with water:



Hudson and Harper (132) studied the attack of different anions at the phosphorus atom. The reactivity was found to be highly dependent upon the basicity towards protons, relative to that of the

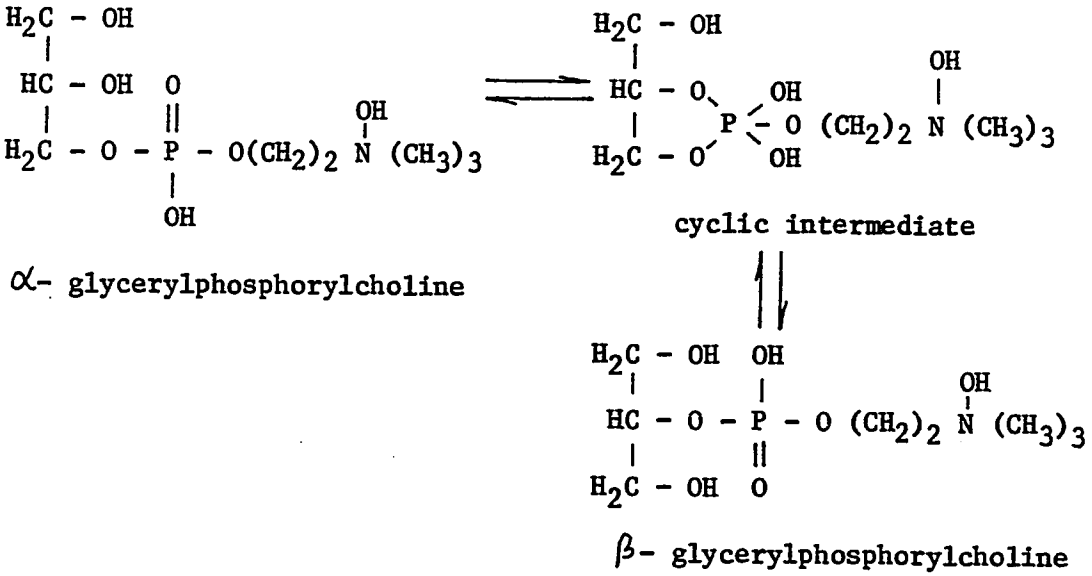
displaced group. The rate constants for the reaction with particular anions (chloride, bromide, thiocyanate, iodide, thiosylphate and hydroxide are calculated and represented. According to these experiments, the hydroxide ion was found to be ca 10^3 times more reactive than the other anions. This reactivity was studied with di- and tri-phosphate esters, which both gave comparable results. The abnormal reactivity of hydroxide ion is attributed to the preferential attack on the phosphorus atom.

The effect of different neighbouring functions on the stability of a phosphate ester toward acid or alkaline hydrolysis deserves special consideration. Different neighbouring groups can radically influence the stability of a phosphate ester. In particular, a neighbouring (vicinal) hydroxyl group can significantly increase the rate of acidic or basic hydrolysis of a phosphate ester. In this process, cyclic phosphates are generally intermediates. These cyclic phosphates are less stable and are easily hydrolyzed with intramolecular transesterification and production of positional isomers.

This effect of a vicinal hydroxyl group is of considerable importance in the study of phospholipids where in many cases a vicinal hydroxyl function can be present either in the glycerol moiety or in the inositol portion of the molecule. One general conclusion can be made regarding the phosphate esters with a vicinal hydroxyl group: There is a "great stability of dialkyl phosphates devoid of vicinal hydroxyl groups. Dialkyl phosphates in which both substituents carry vicinal hydroxyl groups are very unstable and easily hydrolyzed" (Brown, 41). This stability, however, depends also

upon the stereochemistry of the neighbouring function and the nature of the displaced group.

The hydrolysis of phospholipids was also studied by Baer and Kates (18) who postulated the formation of a cyclic orthoester intermediate and observed an $\alpha \rightleftharpoons \beta$ migration of phosphoric acid. The following equilibria in the case of glycerylphosphorylcholine were represented:

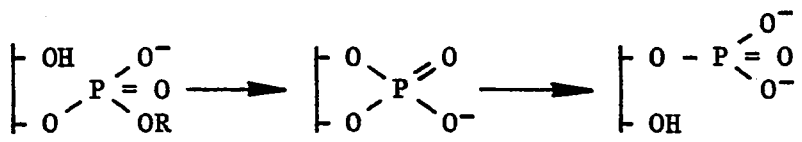


This transesterification reaction was accompanied by a liberation of choline. It was also found that the α-form predominated on acid hydrolysis and the β-form after alkaline hydrolysis. This finding led the authors to conclude that the existence of β-lecithins cannot be justified if β-glycerophosphoric acid was found in the hydrolyzate of a lecithin sample. The β-isomer is regarded as an artifact arising from the phosphate migration during the hydrolytic procedure.

The cyclic 1,2-glycerophosphate was synthesized by Ukita, Bates and Carter (254). It was converted to the more stable barium

salt and characterized in this form. However, these authors failed to obtain evidence on the presence of this compound in the alkaline hydrolyzate of lecithin. Maruo and Benson (187) isolated a cyclic 1,2-glycerophosphate as a product of base-catalyzed methanolysis of glycerophosphatides. They also found that glycerophosphorylcholine was the most labile phospholipid to cyclization and methanolysis whereas the diglycerophosphate and glycerophosphorylinositol were relatively resistant. Extended treatment with KOH in methanol resulted in production of a mixture of glycerophosphate and methyl-glycerophosphate. The same investigators also stated that the cyclic ester is not significantly affected by chromatography with weak acid solvents.

The hydrolysis of phosphate esters with vicinal hydroxyl groups was studied extensively by Brown and his associates (42, 43). It was demonstrated in all the cases studied that the alkaline hydrolysis of such compounds proceeded by an attack of the neighbouring hydroxyl group on phosphorus with expulsion of RO- to give the unstable cyclic diester which then splits to the two isomeric monoesters:

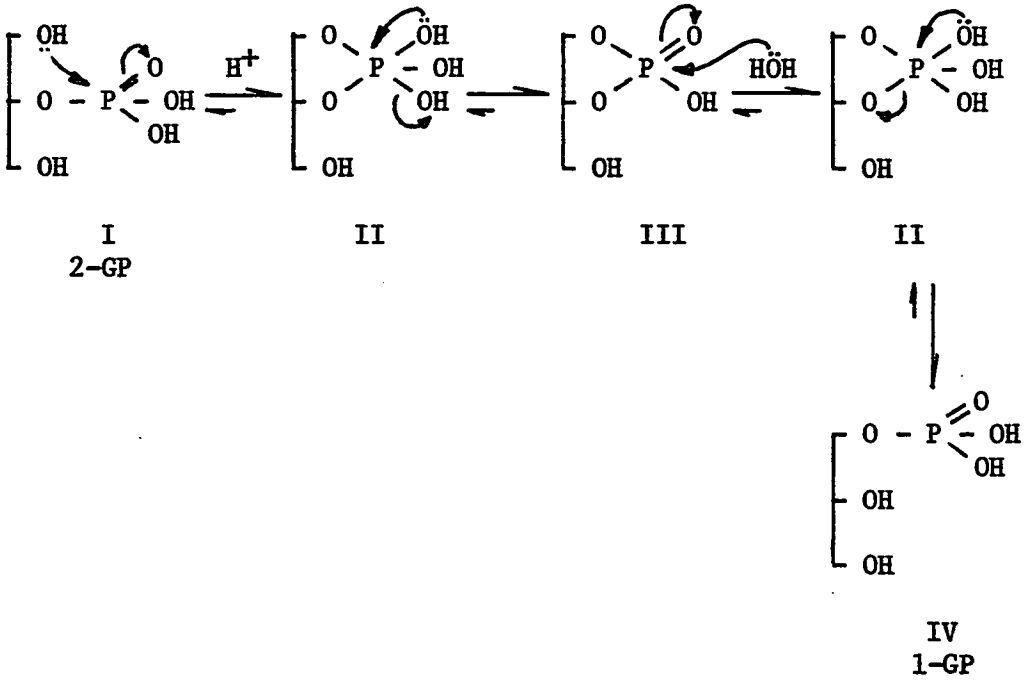


I II III

The RO- group in this case is the group esterified to one hydroxyl of the phosphate, so they concluded that "the participation by the vicinal function results in a much greater lability of the system by comparison with normal dialkyl phosphates which are extremely

stable." Under alkaline conditions, the phosphate group is retained on the hydroxy-alkyl residue and there is no significant production of $RO-PO_3H_2$ from the compound (I). Acid hydrolysis proceeds also via the intermediate cyclic phosphate (II). However, in this case the migration of the alkyl-phosphoryl group to the vicinal position can also take place without loss of the R group.

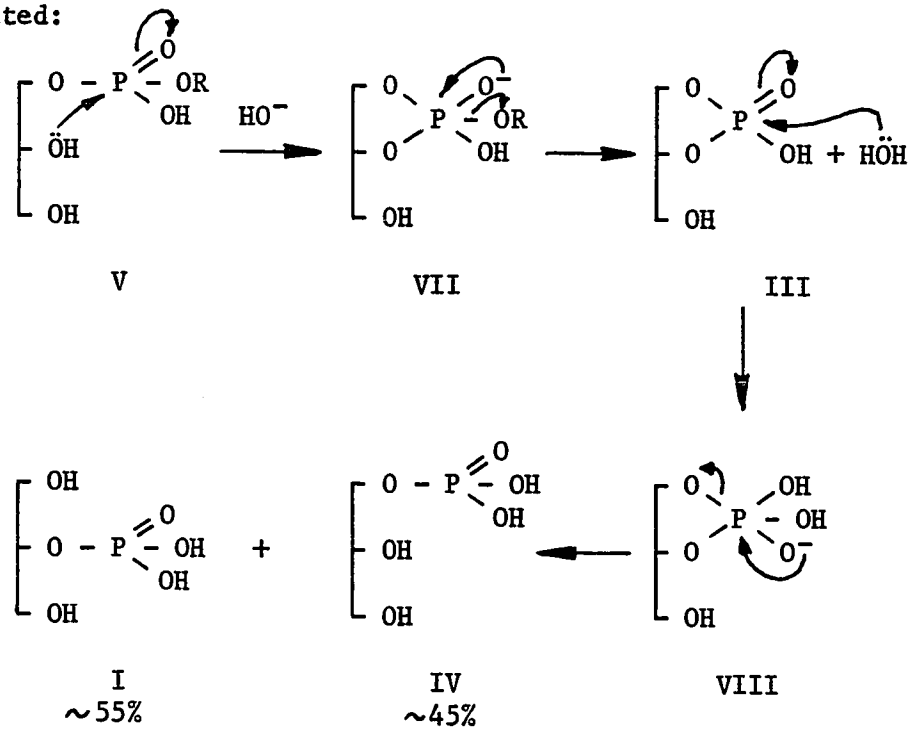
The mechanism of glyceride isomerization in lipid chemistry has been discussed by Serdarevich (233). The mechanism of acid catalyzed isomerization was presented in the following way:



In equilibrium, there is about 85% of the 1-isomer and 15% of the 2-isomer. There are several intermediates in this reaction sequence. Intermediate II is the least stable one, as it involves the highest free energy barrier and it is quickly transformed to the more stable cyclic phosphate III. The product with the lowest free energy is compound IV, which arises from the splitting of III by water and acid.

In this way the whole reaction is thermodynamically controlled. The isomerization of phosphate diesters such as phosphatidylcholine, phosphatidylglycerol, etc. proceeds in the same way, but the alkyl group is lost during the formation of transition state intermediate III.

The following mechanism for base-catalyzed isomerization was presented:



Compared with acid catalyzed isomerization, the quantity of 2-isomer is much greater than in the first case. The latter reaction is, however, kinetically controlled. In this case there is an excess of a less stable product which may be the result of steric or stereo-electronic splitting of the cyclic intermediate. Base-catalyzed hydrolysis and isomerization of phosphoglycerides depends also on the polarity of the medium. This influence was studied by Brockerhoff (39) who found considerably more degradation of the glycerophosphatide

molecule when the hydrolysis was carried out in a less polar medium such as chloroform-ethanol. In a more polar medium (such as methanol-water), less degradation was observed. This phenomenon could be explained by a greater ability of a vicinal hydroxyl group to attack the phosphate group in a nonpolar medium than in a more polar one. In the more polar medium the vicinal hydroxyl group must compete with high electron density of the medium, which results in less degradation of the molecule (233).

MATERIALS AND METHODS

I. CULTIVATION OF BACTERIAL CELLS

The cells of Listeria monocytogenes, which were used for the experiments, belong to strain No. 42, serotype I. This strain was originally isolated by N. F. Stanley from a patient with meningitis (245) and was further used and preserved by E. G. D. Murray. The same strain appears also as McGill strain 42 XXVIII, as classified by Murray.

The bacteria for our experiments were grown at the Swedish Medical Research Council group for Bacteriological Bioengineering at The Karolinska Institute, Sweden by Dr. B. Holmstrom (125). They were grown in batch and continuous cultures at 37°C at a pH 7.4 which was automatically controlled with 5N NaOH. The growth medium was composed of 20 g tryptose, 5 g sodium chloride and 15 g glucose per liter. When the stationary phase of growth was reached, the bacteria were killed by heating either to 120°C for 30 min or to 60°C for 90 min. The cells were centrifuged in Sharples centrifuges or were sedimented in a De Laval separator. They were further lyophilized and shipped in a dry state to our laboratory where they were stored in a cold room at 4°C.

II. EXTRACTION OF LIPIDS

The first operation on the lyophilized listeria cells was the extraction of lipids from this material. For this purpose, the cells had to be disintegrated and this procedure presented some problems

as the cell wall of Listeria monocytogenes has a very rigid structure which is difficult to disrupt by many conventional methods. Sonic disintegration, homogenization in a mechanical cell homogenizer (Braun, Model MKS with 4000 agitating cycles per minute) or compression to 5000 lb/sq. in. and then sudden decompression to atmospheric pressure in a French press, were all unsuccessful. The method developed in our laboratory and published by Carroll, Cutts and Murray (49) gave best results. The method is presented in Fig. 11 and consists of the following operations:

The cells were shaken overnight in a tightly glass-stoppered round bottom flask, together with glass beads, glass powder and $CHCl_3$. The mixture was then filtered through a coarse filter paper and then through a Whatman M-1 paper. The residue was re-extracted several times with $CHCl_3$ in order to free it completely from any lipid material soluble in $CHCl_3$. The resulting $CHCl_3$ extract was mainly composed of diglycerides and free fatty acids but it also contained some phospholipids, as determined by chromatography on acid-treated Florisil (ATF). The main portion of phospholipids was, however, still firmly bound to the membrane material after this extraction and a more polar solvent was necessary to disrupt the lipid-protein bonds.

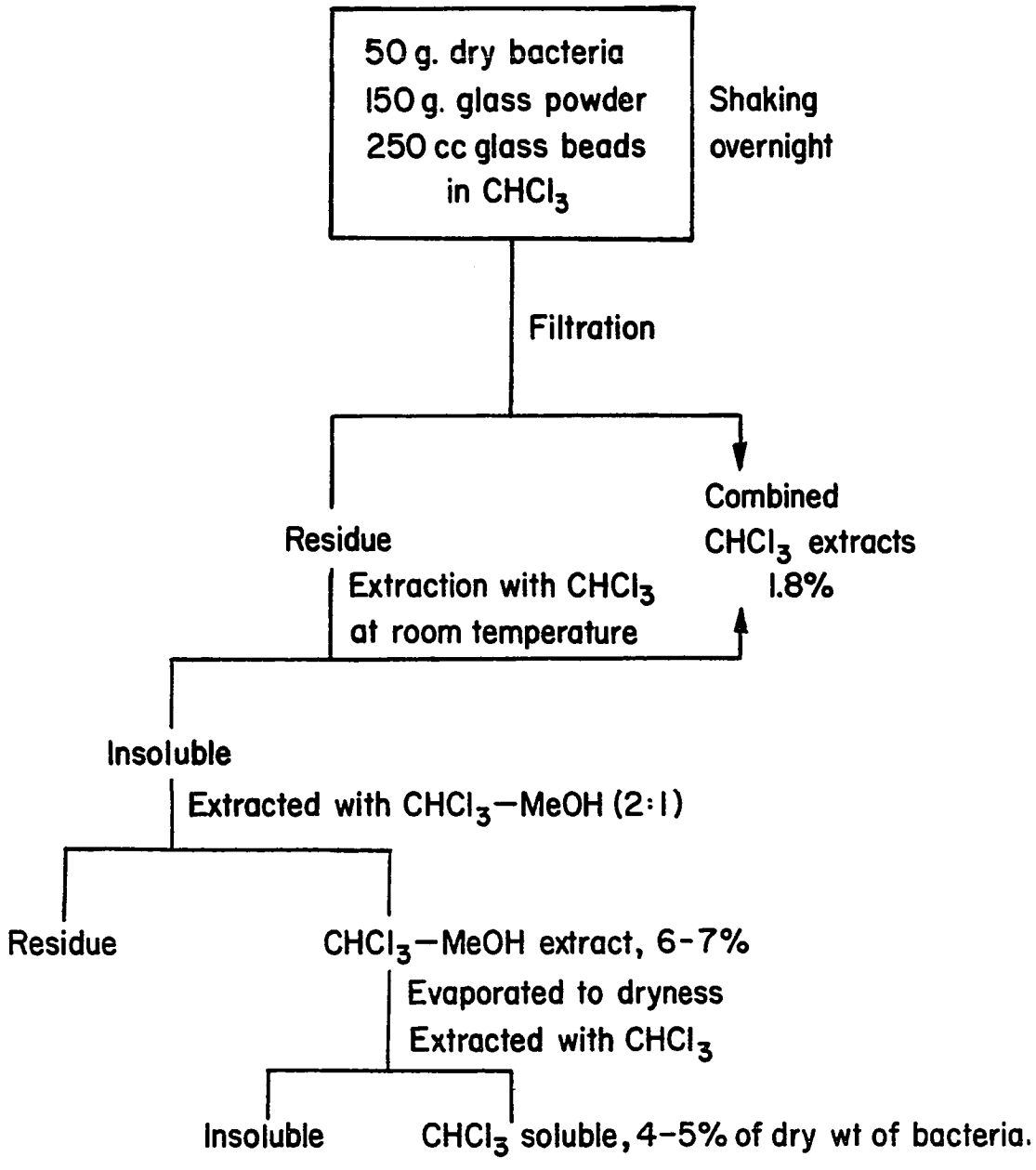
Chloroform-methanol (2:1) was therefore used for extraction of the portion insoluble in $CHCl_3$ and the residue after this extraction was separated by filtration. The extraction was repeated three times and the solvent was left each time for half an hour in contact with the residue. The residue was discarded and the combined $CHCl_3$-MeOH extracts evaporated to dryness and redissolved in $CHCl_3$. This latter

F I G U R E 11

Extraction of lipids from Listeria monocytogenes

Extraction procedure, as used in this study, is illustrated.

EXTRACTION OF LIPIDS FROM LISTERIA MONOCYTOGENES.



procedure was done to eliminate most of the water-soluble material present in the extract. All water soluble material was, however, not completely eliminated by this procedure, as shown by subsequent chromatography on Sephadex G-25 (226). However, as it was assumed that this accompanying material was completely eliminated from the phospholipid samples in the later stages of purification, so that it would not interfere in further analytical work, no attempt was made to eliminate it completely at this stage.

The $CHCl_3$-soluble fraction from the $CHCl_3$-MeOH extract was the material used for further experiments. It was found to be composed mainly of phospholipids, although diglycerides, free fatty acids and a glycolipid were present as well. The material was evaporated to dryness in a vacuum evaporator under nitrogen, weighed, redissolved in $CHCl_3$ containing 10% MeOH and kept in the refrigerator at 4°C.

Some comments concerning this extraction procedure may be appropriate. Since other methods were unsatisfactory for the disruption of the bacterial cell wall and extraction of lipids, the prolonged treatment with glass powder and glass beads in $CHCl_3$ was necessary, but this increased the chance of lipid degradation as well as solubilization of non-lipid material. Hanahan's suggestion (108) of working at room temperature was followed during the extraction; low temperatures were avoided because of the lower solubility of the lipids, and high temperatures could result in considerable degradation of phospholipids. If enzymes, such as phospholipases, were present in the mixture, they could be activated at higher temperatures (108) as well as by the solvents (150).

III. CHROMATOGRAPHY OF THE EXTRACTED LIPIDS

A. Column chromatography on acid-treated Florisil

The extracted lipids were separated by acid-treated Florisil chromatography as reported by Carroll, Cutts and Murray (49). The usual column load was about 1 g of lipids on a 2.5 x 47.0 cm column containing 150 g of acid-treated Florisil. The acid-treated Florisil was prepared by the method of Carroll (47). In this method Florisil is treated with hot HCl in order to eliminate Mg from the Si-O-Mg-OH complex. The resulting material is chemically a silicic acid polymer, similar to commercial silicic acid. The advantage of acid-treated Florisil, compared to most commercially available silicic acid is its coarse mesh size which simplifies the technical operation and permits faster flow rates.

Individual fractions were eluted with different solvents as follows:

Fraction	ml	Solvent
A	600	chloroform
B	600	chloroform-acetone (1:1)
C	600	acetone
D	500	2% methanol in chloroform
E	600	10% methanol in chloroform
F	600	50% methanol in chloroform
G	600	methanol

Usually the chromatography was started in the morning and discontinued after four fractions were collected. The column was left overnight in 2% methanol in chloroform and the chromatography

continued the following morning and finished in the afternoon. Each fraction was usually collected in 2-3 hours. The individual fractions were evaporated to dryness under vacuum, weighed and analyzed by thin-layer chromatography (TLC). The individual phospholipids were separated by preparative TLC and then used for further analytical studies.

B. Anion-exchange chromatography

Anion-exchange chromatography was used for separation and purification of deacylated products obtained by mild alkaline hydrolysis of the separated phospholipids. This was found to be very advantageous since pure deacylated products could be isolated for further analysis. Comparison with standards chromatographed in the same system also provided some qualitative information about the nature of the deacylated products.

The anion-exchange resin was obtained in the chloride form but for use was converted to the formate form by the following procedure:

Dowex 1-X2 anion-exchange resin, 200-400 mesh, in the chloride form, was washed with 6N HCl and then with water until the effluent was acid-free. The resin was then treated with 3M sodium-formate until the effluent was chloride-free and washed finally with water. A formate-borate buffer (0.1M ammonium formate-0.02M ammonium borate, adjusted to pH 9.5 with ammonia) was added to the washed resin and the resin was stored in this buffer in a glass-stoppered bottle.

The column for chromatography (1.0 cm diameter) was packed, to the height of 35 cm, with resin in 0.1M formate - 0.02M borate buffer

at pH 9.5. The material to be separated was dissolved in 0.5 ml of 0.02M ammonium borate, applied to the top of the column, to which then 2.0 ml of the buffer at pH 9.5 were added. The column was eluted with a concentration gradient of formate at pH 8.5. At the start, 150 ml of the buffer was placed in each of the three chambers and the contents of the first two mixing chambers were stirred with magnetic stirrers. The concentration of buffer in the two chambers next to the column was 0.1 M ammonium formate - 0.02 M ammonium borate (pH 8.5) while the third chamber contained a 0.625 M concentration of ammonium formate and 0.02 M borate, also at pH 8.5. The flow rate was 1 drop/15 sec. and 10 ml fractions were collected on a Rinco fraction collector. The individual fractions were analyzed for phosphorus content. The whole setup is represented in Fig. 12.

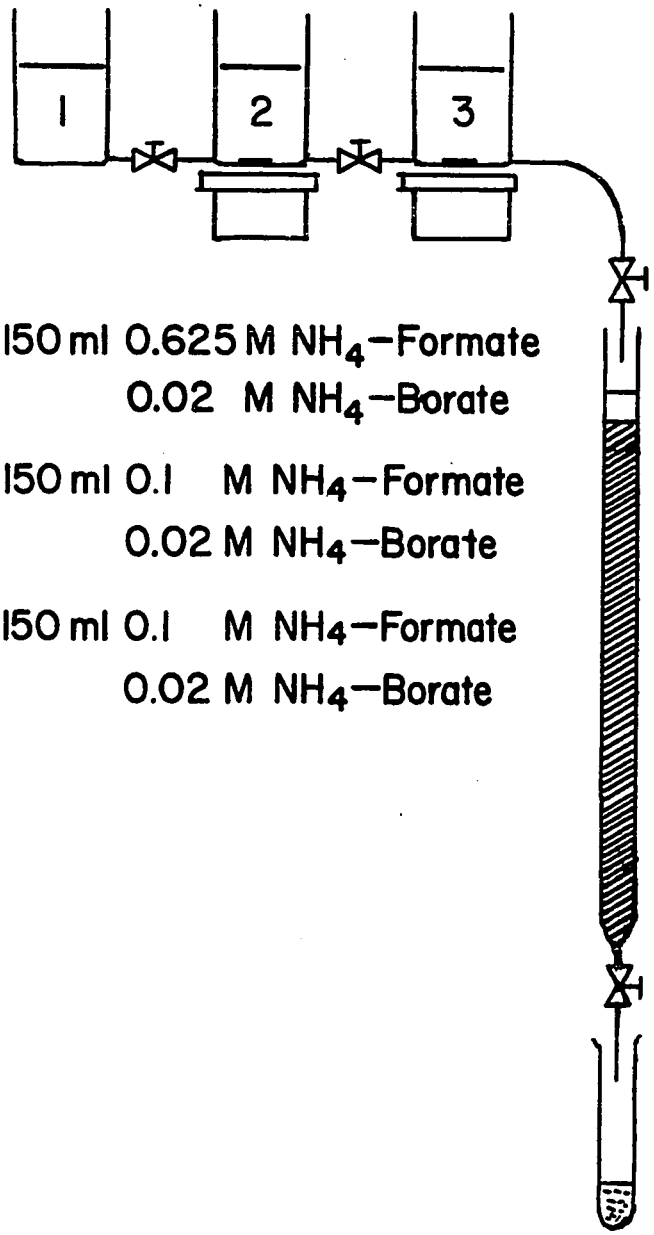
This method was essentially a slight modification of the separation technique used by Wells and Dittmer (268). The difference was in the size of the column, which was shorter and of larger diameter in our case and which resulted in a somewhat greater elution rate. This rate was still low compared to other chromatographic procedures, but it resulted in excellent separation, and, as the samples could be collected without supervision, it was not time consuming. The method used for producing the gradient gave good reproducibility, as tested in several repeated experiments. From 10 to 50 mg was applied on the column and no displacement of the peaks was noticed when different loads in this range were applied.

F I G U R E 1 2

Ion-exchange chromatography of deacylated phospholipids

The general set-up for anion-exchange chromatography is shown.

ION EXCHANGE CHROMATOGRAPHY OF DEACYLATED PHOPHOLIPIDS.



Mixing chambers

Magnetic stirrers

1–150 ml 0.625 M NH_4–Formate
0.02 M NH_4–Borate

2–150 ml 0.1 M NH_4–Formate
0.02 M NH_4–Borate

3–150 ml 0.1 M NH_4–Formate
0.02 M NH_4–Borate

Dowex 1–X2
Anion exchange
resin
200–400 Mesh
31×1 cm

Fraction
collector

C. Chromatography on Sephadex

Although anion-exchange chromatography gave a good separation of the deacylated phospholipids, the pooled, phosphorus-containing samples could not be analyzed as such because they contained large amounts of the ions which came from the buffers used for anion-exchange chromatography as well as from reagents used for mild alkaline hydrolysis. These ions presented difficulty in preparing samples for NMR spectroscopy since the samples had to be dissolved in a limited volume of D_2O. Therefore, attempts were made to eliminate this material from the deacylated phospholipids. As most of the accompanying material was expected to be composed of ammonium formate, attempts were made to eliminate this material by lyophilization in a high vacuum. Some of the material could be eliminated in this way but this was not quantitative. Solvent extraction was also tried with ethanol, methanol and mixtures of chloroform with methanol, but all these procedures were unsuccessful. The method which was finally developed and which proved satisfactory involved chromatography on Sephadex G-15. (G-10 also gave satisfactory results.) For this purpose the resin was allowed to swell in dist. H_2O for 3-4 hours and the slurry was poured into a column to give a volume of resin after sedimentation of 2.0 x 115 cm. Smaller columns, such as 2 x 30 cm and 1.2 x 80 cm were also tried, but as the best separations were obtained with the big column, it was used for all experiments. The material, dissolved in 1 ml of dist. H_2O was applied on the column. The column was eluted with dist. H_2O, 5 ml fractions were collected and analyzed quantitatively for phosphorus and qualitatively for sodium, ammonia

and borate. Flame photometry was used for the sodium and borate, whereas the ammonium ions were assayed with Nessler reagent.

D. Thin-layer chromatography (TLC)

1. Analytical TLC

Glass plates (20 x 20 cm, 20 x 10 cm or 20 x 5 cm) were washed in sodium dichromate-sulphuric acid, rinsed with water followed by methanol and coated with Silica gel H (acc. to Stahl, E. Merck, AG, Darmstadt, Germany) using a Desaga TLC applicator. The coated plates (ca 75 μ thick) were allowed to dry for 0.5 - 1 hour at room temperature and were then activated for two hours in an oven at 120°C. The activated plates were stored in a dust-free, dry atmosphere before use.

Several standards were applied, as seen on the corresponding Figures, representing the developed TLC plates. In some cases a strip application and division of the plate by vertical lines improved the chromatogram, allowed more detailed analysis, and eliminated a concave shape of the front. When the plate was divided in this way, more material could be applied and a more accurate analysis of the individual spots obtained.

The plates were developed, either by a one- or two-dimensional technique, in chromatography jars whose interior sides were lined with a qualitative filter paper. The solvent was added to the jar 15-30 min. before the plate, to allow for equilibration. The solvents used were:

a. For one-dimensional TLC:

Solvent A: $CHCl_3$ - MeOH - H_2O (65:20:3)

Solvent B: $CHCl_3$ - MeOH - 28% ammonia (65:35:5)

Solvent C: petr. ether - ethyl ether - acetic acid (60:40:1)

b. For two-dimensional TLC:

The solvent systems used for these experiments were described by Rouser, Kritchevsky and Yamamoto (226).

Solvent for the first dimension:

$CHCl_3$ - MeOH - 28% ammonia (65:35:5)

Solvent for the second dimension:

$CHCl_3$ - acetone - MeOH - acetic acid - H_2O (5:2:1:1:0.5)

In the case of two-dimensional TLC, the plate was air dried for 10-15 min. until no ammonia could be detected by smell. Only then was the plate placed into the second solvent. After developing, the plates were air dried, sprayed with the appropriate spray reagent and heated at 200°C if necessary.

The following specific sprays were used for detection of lipid spots on thin-layer plates:

i. Sulphuric acid spray for carbon-containing compounds

55% (by weight) sulphuric acid in water containing 0.6% potassium dichromate (224). After being sprayed, the plates were heated in an oven at 200°C for 15-30 min. Black spots appeared due to charring of carbon-containing organic material.

ii. Specific spray for phospholipids

A spray for phospholipids developed by Vaskovski and Kostetski

(258) was used. The spray was prepared by dissolving 16 g of ammonium molybdate in 120 ml of water to give solution I. Eighty ml of this solution was then shaken with 40 ml of concentrated HCl and 10 ml of mercury, for 30 minutes. This gave solution II. Two hundred ml of concentrated H_2SO_4, followed by solution II, was then added carefully to the remainder of solution I. The cooled mixture was diluted with H_2O to 1 liter.

This reagent shows a great specificity for phospholipids and does not react with deacylated phospholipids. Blue phospholipid-containing spots appear immediately after spraying. After 10-15 minutes, a deeper blue colour develops but after more than one hour, the colour in the spots gradually disappears leaving a blue background. The blue colour is more persistant in dark than in light.

iii. Ninhydrin spray

Dry plates were sprayed with a solution containing 0.3 g of ninhydrin in 5 ml of lutidine and 95 ml of n-butanol saturated with water. The plates were dried, after spraying, in an oven at 100°C for about 10 minutes. Red-violet spots appeared on a white background in the case of a positive reaction (238).

iv. Iodine vapour

Iodine crystals were placed in a big covered glass jar. The dry plates were exposed to the iodine vapours for about 1 minute or more. Brown spots appeared quickly after exposure. The colour fades as the absorbed iodine evaporates from the plate (238).

v. Rhodamine spray

This spray was mostly used for preparative TLC. The stock

solution was composed of a 1% Rhodamine 6-G solution in water. The spray was made by mixing 1 ml of the stock solution with 200 ml methanol-water (1:1). Very intense fluorescing, lipid-containing spots could be seen under ultraviolet light.

vi. Ammoniacal silver nitrate

This spray was used for the detection of glycerol and inositol on thin-layer plates. Dry plates were sprayed with a mixture of equal volumes of 0.1N $AgNO_3$ and 7N NH_4OH. A positive reaction is characterized by appearance of dark brown spots after heating the plates at 110°C (238).

vii. Dragendorf reagent

This reagent was used for detection of choline on thin-layer plates and was prepared in the following way (238): Solution I was prepared by dissolving 1.7 g of $Bi(NO_3)_3.5H_2O$ in 100 ml of 20% v/v acetic acid. Solution II was prepared by dissolving 40 g KI in 100 ml water. Dry plates were sprayed with a mixture of 4 ml solution I, one ml solution II and 20 ml dist. H_2O. Purple spots are produced by free choline and orange spots by choline-containing compounds.

2. Preparative TLC

Preparative thin-layer chromatography of lipids was performed in the following way:

The mixture to be separated (10 mg/ml) was applied in a line on a thin-layer plate (20 x 20 cm). The solvent (solvent A or B) was allowed to run to the top of the plate, which was then sprayed either with distilled water or with a Rhodamine 6-G solution. After spraying

with water, white bands were seen, and in the case of Rhodamine spray, the bands were detected under ultraviolet light. The areas of the individual bands were marked and the corresponding bands scraped into separate 100 ml centrifuge tubes which contained $CHCl_3$-MeOH (2:1). Usually the bands from 15 plates were scraped into one set of centrifuge tubes. The silica gel was centrifuged, washed twice with the same solvent and the resulting solution was evaporated to dryness. The residue was weighed and dissolved in 10% MeOH in $CHCl_3$.

In the initial experiments, the plates were sprayed after development with distilled water and the bands were visualized against a black background. However, due to non-uniform elution of the bands and the difficulty of visualizing them, the separations were not very successful and considerable overlapping frequently occurred between adjacent zones. Another disadvantage of this method was that there was always a considerable amount of water on the plate and extraction of the lipids from silica-gel with $CHCl_3$-MeOH resulted in a very wet solvent. The mixture frequently foamed during evaporation and, therefore, required constant attention to avoid loss of material. A more satisfactory procedure was found by using a spray of Rhodamine 6-G in H_2O-MeOH (1:1). A small amount of this spray was sufficient to visualize the individual bands under ultraviolet light. As a result, much less water was present in the extract and very sharp separation of the bands could be achieved. The dye was easily separated by rechromatography of the coloured phospholipid in the same solvent system. The dye always had a higher R_f value than the lipid and it could be seen without any spray, and scraped off the plate.

Its separation from the phospholipid was complete so that it did not interfere in further experiments.

E. Paper chromatography

The deacylated products, previously chromatographed by anion-exchange and Sephadex chromatography, were analyzed by paper chromatography in 7 different solvent systems. The chromatography was performed in tightly covered glass-chromatography jars (30 cm diameter x 45 cm high) on Whatman No. 1 chromatography paper without any pretreatment. The paper was cut in 18 x 46 cm strips on which up to 5 compounds were spotted. The following solvents were used in these experiments:

Solvent No.	Composition	Chromatography	Ref.
1	Isopropanol - 28% ammonia - water (7:1:2)	descending	(163)
2	Tert. butanol - water - trichloroac. ac. (62:38:10%)	descending	(170)
3	phenol saturated with 1% ammonia	descending	(170)
4	butanol - propionic acid - water (5:7:10)*	ascending	(223)
5	propanol - 28% ammonia - water (6:3:1)	ascending	(106)
6	butanol - acetic acid - water (5:4:1)	ascending	(52)
7	1M am. acetate - abs. ethanol (35:65)	ascending	(52)

After development of the chromatograms, which usually took about 24 hours, the paper was dried at room temperature and sprayed with sulphosalicylic acid reagent for phosphorus. White spots appeared

* modified composition

immediately on a mauve background. The reagent was prepared according to Skidmore and Entenman (238) in the following way: 7 g of sulphosalicylic acid and 0.1 g of $FeCl_3.6H_2O$ were dissolved in 25 ml H_2O and the solution was diluted to 100 ml with 95% ethanol.

F. Gas-liquid chromatography of fatty acid methyl esters

The fatty acids in the original phospholipids, in the lyso derivatives obtained after treatment of the original phospholipids with phospholipase A, as well as fatty acids which were liberated with phospholipase A, were all analyzed by gas-liquid chromatography. The chromatograph used was a Barber-Colman gas chromatograph, Model 5340, with a thermal conductivity cell. A non-polar column (3% silicone gum rubber GE SE30 on Chromosorb W) was used and the adsorbent supplied by F. & M. Scientific Corporation, Avondale, Pa, USA. The following temperatures were maintained during chromatography: injector 220°C, column 185°C and detector 255°C. Pure helium was used as the carrier gas.

Before chromatography, the individual fatty acids were converted into the corresponding methyl esters in the following way (49): to a dry sample, weighing about 10 mg, 5 ml of 10% acetyl chloride solution in methanol was added and the methyl esters extracted into the ether phase. The extraction was repeated twice and the ether extracts were combined and washed with distilled water until neutral. The sample was evaporated to dryness under vacuum, dissolved in petroleum ether and injected into the gas chromatograph. The quantitation of the individual acids from the chromatograms was done by multiplying

retention time and peak height according to Carroll (48). Fatty acid methyl ester standards (National Heart Institute, Bethesda, Md., USA) were used for testing the linearity of response in this chromatography.

IV. DEGRADATION OF PHOSPHOLIPIDS

A. Mild alkaline hydrolysis

Mild alkaline hydrolysis of phospholipids was used in this study in order to split the fatty acids from the molecule and obtain a deacylated derivative which could be analyzed further. The convenience of the method is that it gives defined products which can be easily separated and analyzed by methods which could not be applied to the original phospholipids. The products obtained after this treatment are essentially free fatty acids (or their methyl esters) and a deacylated compound which is water-soluble and can, therefore, be separated from the fatty acids and the non-hydrolyzed material. The method is a modification of the classical Dawson method (75) in which a weak solution of NaOH was used for hydrolysis. The procedure followed in our experiments is presented in Fig. 13 and can be summarized as follows:

The sample (100-200 mg) was dissolved in 10 ml of $CHCl_3$ - MeOH (1:2), the solution adjusted to 37°C in a water bath, and 10 ml of 0.1N NaOH in methanol added. The reaction flask was stoppered with a glass stopper, the contents well mixed and incubated for 10 minutes at 37°C. After this time, ethyl formate was added to the incubation mixture until pH 4-5 was reached. Eight ml of $CHCl_3$, 4 ml isobutanol and 8 ml dist. H_2O were then added and the contents shaken for approx.

F I G U R E 1 3

Mild alkaline hydrolysis of phospholipids

The Figure shows the procedure used for partial alkaline hydrolysis.

PARTIAL ALKALINE HYDROLYSIS.

PHOSPHOLIPID
Dissolved in $CHCl_3$–MeOH (1:2)
+ n/10 NaOH in MeOH
10 min at 37°C

+ Ethyl Formate to pH 4–5
+ $CHCl_3$–Isobutanol–H_2O (2:1:2)
shake
centrifuge
wash 2x with MeOH–H_2O (1:2)

AQ. LAYER
(Water soluble glycerophosphates)

$CHCl_3$ LAYER
(Fatty acids and unhydrolysed material)

1 minute. The mixture was then transferred into a centrifuge tube, centrifuged at 2000 rpm for 10 min. and the upper layer pipetted into a 25 ml volumetric flask. The chloroform layer was washed twice with 10 ml MeOH-H_2O (1:2) and the upper layers combined into the volumetric flask. The washed chloroform layer was transferred into another 25 ml volumetric flask, both flasks were filled with appropriate solvent to the mark, and equal aliquots pipetted in triplicate for phosphorus analysis. The ratio of water-soluble to total phosphorus was used as a measure of the hydrolysis process.

The water-soluble hydrolysis products were recovered from the water-methanol phase by concentrating it under vacuum to remove methanol and then lyophilizing the remaining solution. The lyophilized material was further purified by anion-exchange chromatography, as described earlier. Fatty acids for gas-chromatographic analysis were recovered from the chloroform phase.

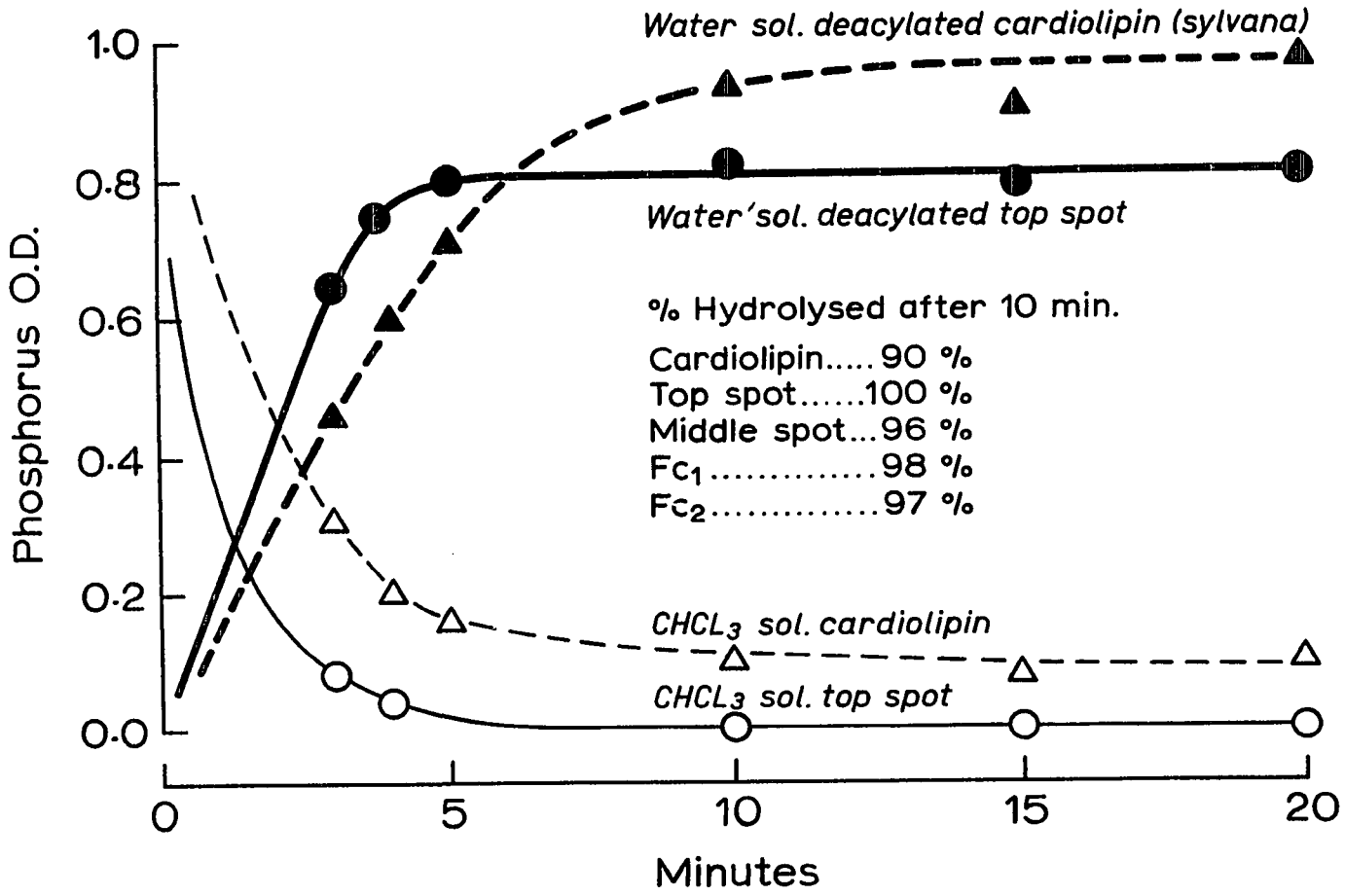
Some comments about this method are essential. It was of particular interest to hydrolyze off only the fatty acids and not to degrade or alter the backbone of the original phospholipid molecule. As noticed by many investigators (9, 39, 131), a prolonged hydrolysis as well as high alkaline concentration can result in phospholipid degradation. Therefore, a low concentration of alkali and a short period of hydrolysis was used. The final concentration of NaOH in the reaction mixture was 0.05N and the time necessary for complete hydrolysis was studied for our case. As seen in Fig. 14, a cardiolipin standard (B.H.CL.) and the top spot (Fa) showed somewhat different rates of hydrolysis. Whereas 100% hydrolysis of the top spot was observed after 5 min.

FIGURE 14

A time study of alkaline hydrolysis of phospholipids.

A time study of alkaline hydrolysis of beef heart cardiolipin standard and Fa is presented. The phosphorus content in the water-and chloroform soluble phase after hydrolysis is indicated. The % of hydrolysis for cardiolipin standard, top spot (Fa), middle spot (Fb) and bottom spots (Fc_1 and Fc_2), after 10 minutes is also presented. Further details are given in the text.

ALKALINE HYDROLYSIS
OF PHOSPHOLIPIDS FROM L. MONOCYTOGENES.
(0.1 N NaOH in MeOH, 37°C).



of incubation, beef heart cardiolipin was only about 80% hydrolyzed at this time. As the other compounds (Fb, Fc_1 and Fc_2) were almost completely hydrolyzed after 10 minutes, this time was chosen for all experiments.

Another point, which should be mentioned here, is the treatment of the mixture after the hydrolysis was finished. Dawson (75) used a cation-exchange resin or ethyl formate to neutralize the excess alkali and convert the sodium salts of fatty acids to free fatty acids. As reported by Hubscher et al. (131), there was a considerable loss of phosphate due to adsorption if ion-exchange resin was used at this stage. Ethyl formate was therefore used in our experiments. This resulted in a mild acid medium which allowed the conversion of sodium salts to the free fatty acids (or their ethyl esters) with production of sodium formate, which did not interfere in further analysis.

The polarity of the solvent in which the hydrolysis is performed can have a marked influence on the stability of the phosphate ester bonds. According to Brockerhoff (39), there is considerable breakdown of phosphate esters in a non-polar medium. He studied this breakdown in solvent systems with different polarities and found almost 100% breakdown of lecithin in chloroform, whereas increasing concentrations of methanol in chloroform resulted in decreased breakdown. With $CHCl_3$-MeOH (2:3) and 0.1M NaOH, only 1.6% of the phosphate bonds were split. Based on these observations, the concentration of chloroform in the NaOH incubation mixture was kept as low as possible. The sample was dissolved in $CHCl_3$-MeOH (1:2) and with the addition of methanolic NaOH the final concentration of chloroform was further diminished.

Because the hydrolyses were conducted in a medium containing limited amounts of non-polar solvent and a low concentration of alkali, and because of the short period of hydrolysis and the mild neutralization procedure, no considerable breakdown of the phospholipid molecule was expected during this treatment. The small quantity of artifacts, detected by subsequent anion-exchange chromatography, supported this prediction.

B. Acetic acid hydrolysis

Acetic acid hydrolysis was also used in analysis of the structure of the isolated phospholipid molecules. In particular, the hydrolysis products from the Fa compound were compared with those obtained by hydrolysing a cardiolipin standard as elaborated by Coulon-Morelec, Faure and Marechal (67).

The sample to be hydrolyzed (ca 10 mg) was evaporated to dryness in a glass-stoppered test tube, 1 ml of 90% acetic acid was added and the tube was stoppered and placed in a boiling water bath. After different time intervals, the tubes were cooled, 0.5 ml dist. H_2O and 2 ml $CHCl_3$ were added and the tubes well shaken for ca 1 min. After centrifugation, the two phases were separated, the water phase washed twice with 2 ml $CHCl_3$, and the combined chloroform phases finally washed with 2 ml H_2O. All water and chloroform layers were transferred into 10 ml graduated cylinders or volumetric flasks, an aliquot pipetted in triplicate from each flask and used for determination of phosphorus. The remaining water and chloroform layers were evaporated to dryness under vacuum and analyzed by paper and thin-layer

chromatography, respectively. The rate of hydrolysis was determined by the ratio of water-soluble to total phosphorus content of the original sample.

C. Enzymatic degradation

Phospholipases A, C and D were used specifically to degrade the various phospholipid molecules. Their specificities are presented in Fig. 4. Most of the experiments were done with phospholipase A, which was commercially available from snake venom and which split specifically the fatty acids from the β-position of the phospholipid molecules. These fatty acids could be separated and analyzed by gas-liquid chromatography. The other product was a lyso derivative which had less fatty acids than the original phospholipid. These lyso derivatives were also analyzed for fatty acids, as well as chemically for glycol, phosphorus and fatty acid ester groups. In this way, the products obtained after treatment with phospholipase A gave information about the distribution of fatty acids and also presented further support for the proposed structures of the isolated phospholipids.

1. Treatment with phospholipase A

Phospholipase A was prepared from snake venom (lyophilized Ancistrodon piscivorus piscivorus venom, Ross Allen's Reptile Institute, Inc., Silver Springs, Fla) according to the procedure developed by W. L. Magee (personal communication), in the following way:

Fifty mg of venom were dissolved in a centrifuge tube in 5 ml

of water and the pH was adjusted to 4.5 with 0.1 N HCl, using a pH meter. The temperature of the solution was then brought to 75°C, maintained for 5 minutes and the solution then rapidly cooled to room temperature. The cooled solution was centrifuged at 2000 rpm for 10 minutes, the supernatant decanted into a 50 ml centrifuge tube and 15 ml of cold (-15°C) acetone added. After mixing, the sample was stored at -15°C for 1 hour and then centrifuged. The supernatant was discarded, the precipitate washed once with cold acetone, centrifuged, dried and stored in an evacuated desiccator at room temperature. The total yield was ca 20 mg with the reported specific activity of 5000 mμEq acyl ester linkage (in phosphatidylcholine) hydrolyzed per minute per mg protein.

The sample was incubated with the enzyme in a collidine buffer system according to Magee and Thompson (180). The collidine buffer was prepared as follows:

Two ml of 0.005 M $CaCl_2$ were mixed with 0.33 ml of γ-collidine (2, 4, 6-trimethylpyridine) and 20 ml of dist. H_2O were added. The mixture was vigorously shaken for 1-2 minutes and the pH adjusted to 7.3-7.4 with 1N HCl. The solution was then made up to 25 ml and used immediately.

The following incubation procedure was used:

About 10 mg of the substrate was placed in a 25 ml glass-stoppered Erlenmeyer flask, the solvent evaporated under vacuum at room temperature and 2.0 ml of collidine buffer added. The contents were sonicated for several minutes until a uniform emulsion resulted without noticable lipid adhering to the walls of the flask. The

flask was cooled in ice during this procedure. Ether (0.2 ml) was added to the sonicated mixture, followed by 1-2 mg of the phospholipase A preparation which was previously dissolved in 0.1 ml of 5 mM $CaCl_2$. The flask was tightly stoppered and placed in an incubator at 30°C and shaken gently for 40-48 hours. After this period, the ether was evaporated under nitrogen, the contents transferred into a glass-stoppered test tube (1.0 x 15 cm) to which 2.0 ml of $CHCl_3$ and 1.0 ml of MeOH were added. The contents were shaken for 1 minute, the phases separated and the water-methanol phase washed twice with 2 ml $CHCl_3$. All chloroform extracts were combined, concentrated and analyzed by TLC. The free fatty acids liberated with phospholipase A were separated from the lyso derivatives either by preparative TLC in a $CHCl_3$-MeOH-H_2) system (solvent A) or on a 1.0 x 20.0 cm column, packed with acid-treated Florisil in chloroform. The fatty acids were eluted with chloroform and the lyso-derivatives with $CHCl_3$-MeOH (1:1). The free fatty acids as well as the acids from the lyso derivatives were analyzed by gas-liquid chromatography as described on page 98.

2. Treatment with phospholipase C

Enzymatic degradation of the isolated phospholipid molecules with phospholipase C was also investigated. This enzyme is expected to liberate a diglyceride from PG or DPG, and this could be characterized in the organic phase after extraction from the enzymatic incubation mixture. For our experiments, a commercial preparation from Cl. welchii was used. The enzyme preparation was supplied by Sigma Chemical Company, St. Louis, Missouri, USA. The procedure is essentially a modification of the method used by Haverkate, Houtsmuller and

van Deenen (115). A chloroform solution, containing about 10 mg of substrate and 1.0 mg of egg yolk lecithin, was evaporated to dryness 2 ml of Tris buffer at pH 7.38 were added and the mixture sonicated for a few minutes to obtain a uniform emulsion. Approximately 5 mg of phospholipase C in 0.2 ml of 5 mM $CaCl_2$ was added, followed by 2 ml of ether. The reaction flask was tightly stoppered with a glass stopper and the contents shaken moderately for 2 to 20 hours at room temperature. The phases were separated by the method used for phospholipase A hydrolysates. The two layers were placed in 10 ml volumetric flasks and aliquots taken for phosphorus determinations. The rate of hydrolysis was calculated by the ratio of water-soluble to total phosphorus after subtraction of the blank values. A blank sample, which contained 1.0 mg of egg yolk lecithin was treated in the same way as the analyzed sample. The $CHCl_3$ phase was concentrated and analyzed by TLC in a petroleum ether - ether - acetic acid system (solvent C). It is interesting to note that the enzyme was only active in the case of Fc_1 and that the addition of egg yolk lecithin was essential. Addition of pork lecithin instead of egg yolk lecithin did not have any effect on the hydrolysis and the rate was very low or almost negligible.

3. Treatment with phospholipase D

As shown in Fig. 4, phospholipase D is expected to liberate phosphatidic acid, both from PG and DPG. However, this could not be demonstrated in either case. The method which was used in these experiments was that of Haverkate and van Deenen (114). The source of the enzyme was cabbage (Sigma Chemical Company).

Ten mg of phospholipid was incubated with an equal amount of the crude enzyme in 2 ml of acetate buffer at pH 5.6, to which 2 ml of ether were added. The mixture was shaken vigorously for four hours at 30°C and the water and chloroform phases were separated as explained in the experiments with phospholipase A. The chloroform-soluble portion was analyzed by TLC in solvent A and in solvent C.

V. CHEMICAL ANALYSIS

The original phospholipids, their deacylated products and their lyso derivatives obtained by enzymatic degradation, were all quantitatively analyzed. In the case of the original phospholipids, fatty acid ester groups, phosphorus, glycol groups and glycerol were determined, whereas in the deacylated products, phosphorus and glycol were analyzed. In the lyso derivatives, ester groups, phosphorus and glycol were determined. The methods involved were taken from the literature but their reproducibility was tested in each case with standard samples. Standard samples were also included with every series of experiments and were found to give excellent reproducibility and to follow Lambert-Beer's law within the limits of their use.

A. Determination of phosphorus

Phosphorus was determined by a modified Ernster method (82). The method was essentially the following:

An aliquot (0.05-0.5 ml) containing 1-10 μg P was pipetted into a glass-stoppered test tube (1.5 cm O.D. by 15 cm long) and 1 ml of 70% perchloric acid was added. After addition of several grains of

anti-bumping granules, the tubes were placed on a hot plate and the contents boiled for ca 1 hour or longer, if necessary, to clear the contents completely. After the digestion was completed, the tubes were cooled and 4 ml of distilled water added to each, followed by 5 ml of an isobutanol-benzene (1:1 v/v) mixture. One-half ml of NH_4-molybdate (10% water solution) was then added to each tube, the tubes stoppered with a glass stopper and shaken vigorously for at least one minute. Easily separable layers, which should not be turbid, are an indication that the extraction of the phosphate-molybdate complex into the upper phase is complete. After the layers were completely separated, 4 ml from the upper layer were pipetted into a clean colorimeter tube to which 4 ml of acid alcohol (6.6 ml conc H_2SO_4 + 200 ml abs. ethanol) were added. A clear blue colour was then developed by addition of 0.5 ml $SnCl_2$, freshly prepared by dilution of 1 ml of a stock solution to 200 ml 1N H_2SO_4. (The stock $SnCl_2$ solution was prepared by dissolving 10 g of $SnCl_2$ in 25 ml conc. HCl.) After full development of the colour, which takes approximately 30 minutes, the optical density was recorded at 730 mμ using a Coleman Junor II, Model 620 Spectrophotometer. The readings were done against a reagent blank. The standard curve was prepared with each set of determinations using 5-10 different concentrations of KH_2PO_4 in the range of 1-10 μg of phosphorus.

Determination of phosphorus was shown to be highly reproducible by this method. It involved a relatively long procedure, but as many parallel samples (up to 40) could be handled at the same time, complete analysis of a column with 30 to 35 fractions could be finished in less

than a day. In the case of analysis of many column fractions, the procedure was always done in this way: all samples were analyzed at the same time and then the analysis of the samples which showed a positive reaction was repeated in duplicate, including in each case at least one sample from each side of the peak. If the duplicate results corresponded to the preliminary ones, no further determinations were done; but if there was a considerable discrepancy, another sample was analyzed in duplicate. In this way, the results presented for the column fractions represent a mean value of a least three determinations.

In connection with the analysis for phosphorus, there are some points which deserve comment. The extraction of the phosphorus-molybdenum complex into the organic phase should be quantitative. A good indication is that the layers separate easily and a clear water layer results. If this point is overlooked, considerable error can arise. The digestion should also be complete and the temperature should be adjusted so that none of the samples run dry during digestion. Molybdate was found to give satisfactory results when used even two weeks after preparation but considerable source of error could arise if the $SnCl_2$ solution is not fresh. The stock solution was shown to be stable for at least two months if kept in a refrigerator, but the reducing $SnCl_2$ solution should be prepared immediately before use, as Sn^{++} easily oxidizes to Sn^{++++}, in which form its reducing property is completely lost. The developed colour is clear blue and is stable for a few hours. In this work the chosen method was preferred to the method in which ascorbic acid is used as a reducing agent. The developed colours were not so distinct in the latter case, and the

higher sensitivity of the latter method was of no advantage in our experiments.

B. Determination of glycerol

The determination of glycerol in phospholipids is a difficult procedure. Before the actual analytical procedure can be applied to such compounds, the glycerol must be quantitatively removed from the phosphate group and this procedure usually involves acid hydrolysis of the corresponding phospholipid. Some preliminary experiments were performed to determine the best method of achieving complete degradation of the glycerophosphate molecule without degradation of the glycerol itself. Renkonen reported (219) good hydrolysis of phospatides without degradation of glycerol, using 2N HCl for 48 hours at 125°C, but Courtade, Marinetti and Stotz (68) found that under these conditions the glycerol in their samples was considerably degraded, resulting in low glycerol values. To find the optimum HCl concentration for our glycerol assay, four compounds (α-GP, B.H.Cl, Fa and Fc_1) were subjected to hydrolysis with different concentrations of HCl, ranging from 0.3 to 6 normal. It was found that the optimum concentration for these compounds was 2N HCl, and this normality of the acid was therefore used for all glycerol determinations. The procedure was essentially the following:

A sample containing 0.2-1.0 μmoles of glycerol was placed in a Pyrex-glass test tube (1.5 x 15 cm) and the solvent evaporated under nitrogen. Five ml of 2N HCl were added, the tube sealed under vacuum and placed in an oven set at 125°C, where it was kept at this

temperature for 48 hours. The acid hydrolysate was then transferred into a glass-stoppered test tube. Two ml $CHCl_3$ were added, shaken, and two samples of 2 ml each were pipetted from the water layer and used for glycerol assay.

To the two milliliter aliquots was added 0.1 ml of 10N H_2SO_4, followed by 0.5 ml of 0.1M $NaIO_4$. The contents were mixed and stored at room temperature for five minutes, and the reaction then stopped by adding 0.5 ml of 10% $NaHSO_3$. For estimation of formaldehyde, produced by periodate oxidation, 0.5 ml were withdrawn from the oxidation mixture into a clean glass-stoppered test tube. Five ml of 0.18% chromotropic acid (1, 8-dihydroxynaphthalene-3, 6-disulphonic acid, BDH laboratory reagent) in 20N H_2SO_4 were added to the tube, which was then stoppered and incubated in a boiling water bath for 135 min. The optical density was determined at 570 mμ on a Coleman Spectrophotometer, against a reagent blank. A standard curve, made with analytical grade glycerol, treated in the same way as the sample, was included in each set of determinations and used for the calculation of the glycerol content.

C. Determination of glycol groups

The method of Ansell and Spanner (10) was used for this determination. The sample to be analyzed (0.5-1.0 μmole glycol) was evaporated to dryness in a glass-stoppered test tube and then dissolved in 0.25 ml of 95% ethanol. To this solution, 0.25 ml of a 1% solution of periodic acid (H_5IO_6) in 95% ethanol was added, the tube stoppered and left for 60 minutes at room temperature. The reaction mixture was

then diluted with 0.75 ml dist. H_2O, followed by 0.375 ml of 1N HCl. Aqueous 1.2N sodium arsenite (0.125 ml) was then added and the whole mixture diluted with dist. H_2O to 2.5 ml. An aliquot of 0.5 ml was pipetted for formaldehyde determination, 9.5 ml of chromotropic acid added, the tubes stoppered and placed in a boiling water bath for 30 minutes, avoiding direct exposure to light. Chromotropic acid was prepared by dissolving 1.0 g of 1, 8-dihydroxynaphthalene-3, 6-disulphonic acid in 100 ml dist. H_2O to which 12.5M H_2SO_4 was added to a total of 500 ml. This solution, as well as other reagents, was always freshly prepared before use. A standard curve was prepared with batyl alcohol [$CH_3(CH_2)_{17}.O.CH_2-CHOH-CH_2OH$] obtained from the Sterling-Winthrop Research Institute, Rensselaer, N.Y.

D. Determination of fatty acid ester groups

The spectrophotometric method of Rapport and Alonzo (216) was used for this determination. The method requires little time and gives good reproducible results.

Reagents:

1. Reagent grade absolute ether
2. Ethanolic hydroxylamine hydrochloride (3% solution in 95% ethanol)
3. Ethanolic NaOH (3% solution in 95% ethanol)
4. Alkaline hydroxylamine solution (equal volumes of 2. and 3. are mixed, filtered after 5 minutes and the filtrate used)
5. Ethanolic acid ferric perchlorate (prepared by dissolving 0.5 g ferric perchlorate in a solution containing 487 ml of 95% ethanol and 13 ml of 70% perchloric acid)

Reagent 5 was stored in the refrigerator and was stable for at least 2 weeks, whereas reagents 2, 3 and 4 were prepared immediately before use.

Procedure:

A sample of phospholipid was introduced into a 1.5 x 15 cm glass-stoppered test tube and the solvent removed by evaporation. To the dry residue, 3 ml ether were added and the tube was swirled to dissolve or suspend the material. Then 0.1 ml of the alkaline hydroxylamine solution (soln. No. 4) was added. After mixing, the solvent was evaporated under vacuum and 6 ml of acid ferric perchlorate solution (soln. No. 5) were added. After 30 minutes the optical density at 530 mμ was recorded on a Coleman Spectrophotometer. A reagent blank was used and a standard curve made with known concentrations of methyl stearate. Good readings were obtained between 0.1 and 0.3 μmole of ester.

VI. SPECTROSCOPIC METHODS

A. Infrared Spectroscopy

The phospholipids isolated by preparative TLC were analyzed by IR spectroscopy. The sample to be analyzed was first completely freed of solvent and then dissolved in spectranalyzed $CHCl_3$ and placed in a 0.1 mm NaCl IR-cell. A reference of spectranalyzed $CHCl_3$ was used with each sample. The spectra were recorded on Beckman IR-10 and Beckman IR-5A Infrared Spectrophotometers (Beckman Instruments, Fullerton, California). A polystyrene film was used as a reference and the peak at 6.246 microns or 1601.0 wavenumber in cm^{-1} was recorded on

the spectra.

B. NMR Spectroscopy

Nuclear magnetic resonance spectroscopy was found to be particularly useful for analysis of the structure of water-soluble products obtained by deacylation of the phospholipids isolated by preparative TLC. The spectra were recorded on a Varian A-60 NMR-spectrometer and the spectrum of deacylated Fc_2 was also recorded on a Varian HA-100 NMR-spectrometer. Both instruments were manufactured by Varian Associates, Palo Alto, California, USA. The samples (20-50 mg) were dissolved in 0.3 ml D_2O. The proton resonance of an external standard of tetramethylsilane was recorded in each case.

VII. EXPERIMENTS WITH LABELLED PHOSPHATIDYLGLYCEROL

A standard sample of phosphatidylglycerol (gift from N. Z. Stanacev, University of Toronto, Dept. of Pathological Chemistry) labelled with tritium according to a procedure published by Stanacev, Chang and Kennedy (243) was co-chromatographed with bacterial phospholipids in a 2-dimensional solvent system. The TLC plate was divided after development into 324 squares and the silica gel of the 141 middle squares scraped separately into counting vials. Twenty-five ml of the scintillation counting solution [0.55% 2, 5-diphenyloxazole (PPO) + 0.02% 1, 4-bis-(2-5-phenyloxazolyl)benzene (POPOP) in toluene] was added to each vial and the tritium activity measured in an automatic scintillation counter (Packard Model 3375 TRI-CARB Liquid Scintillation Spectrometer). Each sample was counted

three times for 20 minutes each time, and the mean specific activity as counts/minute reported. The background was automatically subtracted from the readings.

RESULTS

I. EXTRACTION OF LIPIDS FROM BACTERIA

Several fractions were obtained after extraction of lipids from disrupted bacterial cells. The combined chloroform extract (Fig. 11) amounted to ca 1.8% of the total dry weight of bacteria. This extract was composed mainly of non-polar lipids, (diglycerides and free fatty acids) but it also contained phospholipids. The chloroform-methanol extract normally made up 6 - 7% of the dry weight of bacteria and re-extraction with chloroform gave a chloroform-soluble portion amounting to 4 - 5% of the dry weight. This portion was composed mainly of phospholipids and represented the material used for further analysis.

II. ISOLATION AND ANALYSIS OF PHOSPHOLIPIDS

A. Column chromatography

Both the chloroform extract and the chloroform-soluble fraction from the $CHCl_3$-MeOH extract were chromatographed on an acid-treated Florisil (ATF) column. The chloroform extract (900 mg) was chromatographed on one column with the results shown in Table I. The chloroform-soluble portion (2.0 - 2.5 g) from one extraction batch was chromatographed on two columns. Typical results from one column, on which 1.021 g of material was applied, are represented in Table II.

B. Thin-layer chromatography (TLC)

All lipid fractions obtained by column chromatography were

TABLE I

Acid-treated Florisil chromatography of $CHCl_3$ extract of the lipids from Listeria monocytogenes

Fraction	ml of eluent	Eluent	Recovered mg	Recovered %
A	600	$CHCl_3$	519.0	57.7
B	600	$CHCl_3$-acetone (1:1)	100.5	11.2
C	600	acetone	72.8	8.1
D	500	$CHCl_3$-2% MeOH	19.2	2.1
E	600	$CHCl_3$-10% MeOH	30.8	3.4
F	600	$CHCl_3$-MeOH (1:1)	121.5	13.5
G	600	MeOH	14.1	1.5
		Total:	877.9	97.5

TABLE II

Acid-treated Florisil chromatography of the $CHCl_3$-soluble portion from $CHCl_3$-MeOH extract of the lipids from L. monocytogenes

Fraction	ml of eluent	Eluent	Recovered mg	Recovered %
A	600	$CHCl_3$	105.6	10.4
B	600	$CHCl_3$-acetone (1:1)	46.3	4.5
C	600	acetone	112.7	11.1
D	500	$CHCl_3$-2% MeOH	15.0	1.5
E	600	$CHCl_3$-10% MeOH	85.9	8.4
F	600	$CHCl_3$-MeOH (1:1)	527.4	51.7
G	600	MeOH	58.4	5.7
		Total:	951.3	93.3

analyzed by TLC (Figs. 15 and 16).

The non-polar lipids (NL) in fraction A (Table II) were chromatographed in a petroleum ether-ether-acetic acid system and the results are shown in Fig. 15, Lane 2. Standards of monoglyceride (MG), 1, 3-diglyceride (DG), free C_{15} anteiso acid (FA) and tripalmitin (TG) are included in this Figure.

Fig. 16 shows the results obtained by chromatographing all of the different column fractions in a chloroform-methanol-water system. The acetone fraction (C) contains a fairly pure glycolipid which was separately characterized as diglycosyldiglyceride. (P. W. Deroo and K. K. Carroll, unpublished data). The portion eluted with 10% methanol in chloroform contained some phospholipid with a fairly high R_f value which is referred to as top spot or Fa. The main quantity of the phospholipids, eluted with 50% methanol in chloroform was composed of three main components: top spot or Fa, middle spot or Fb and bottom spot or Fc (Fig. 16). When the three components were separated by preparative TLC and analyzed separately in a $CHCl_3$-MeOH-NH_4OH system, it could be seen that Fa and Fb still ran as single spots, whereas Fc was separated into two distinct components, Fc_1 and Fc_2. The same separation was also seen on a two-dimensional TLC plate (Fig. 17) so that there existed four major, distinctly separable, phospholipids in the lipid extracts of Listeria monocytogenes. The relative proportions of these lipids, isolated from fraction F of the $CHCl_3$-soluble material are represented in Table III.

The phospholipids separated by one- and two-dimensional TLC were also sprayed on the plate with specific reagents. They all

F I G U R E 1 5

Thin layer chromatogram of fraction A from chloroform-soluble portion from the chloroform-methanol extract

Fraction A, eluted from ATF column with $CHCl_3$ is compared on a TLC plate with authentic standards. The plate is developed in petr. ether-ether-acetic acid (60:40:1) and the spots visualized by H_2SO_4 spray.

MG - monoglyceride standard

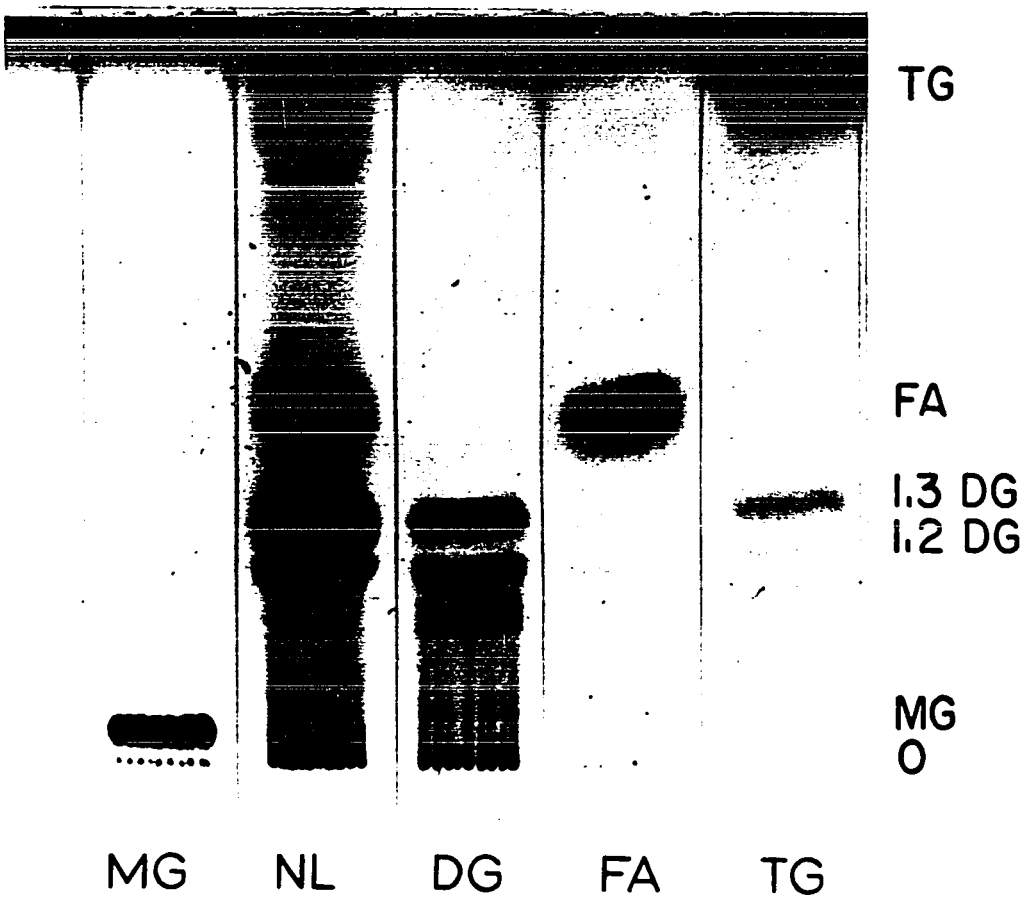
NL - fraction A

DG - 1, 3-diglyceride standard

FA - free fatty acid (C_{15} anteiso) standard

TG - triglyceride standard (tripalmitin)

NEUTRAL LIPIDS
OF L. MONOCYTOGENES



F I G U R E 1 6

Lipids of Listeria monocytogenes (TLC chromatograms)

The lipids separated by ATF chromatography and preparative TLC are presented on the two TLC chromatograms. The left chromatogram was developed in solvent A (C-M-W) and the right in solvent B (C-M-AM). The spots are visualized by H_2SO_4 spray.

solvent A - $CHCl_3$-MeOH-H_2O (65:20:3)

solvent B - $CHCl_3$-MeOH - 28% ammonia (65:35:5)

Fa, Fb, Fc- phospholipids preparatively separated in solvent A

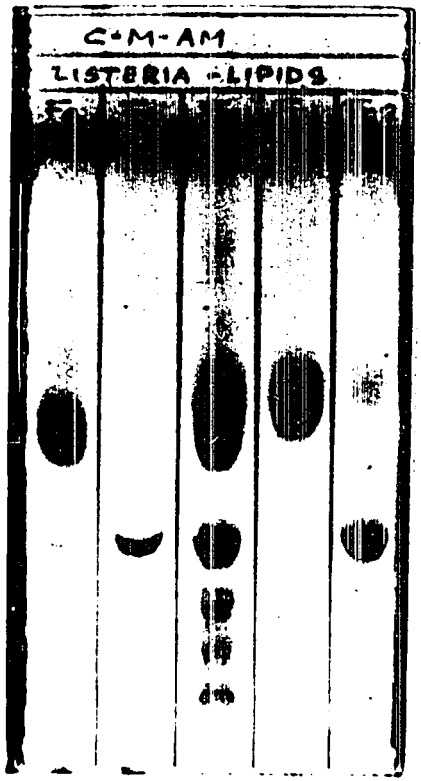
Fc_1, Fc_2 - phospholipids preparatively separated in solvent B

Further details in the text.

LIPIDS OF LISTERIA MONOCYTOGENES

C-M-W

C-M-AM



Fraction	A	B	C	D	E	F	G	Fa	Fb	Fc
Eluent	Chl.	Ac.	Ac.	Me.	Me.	Me.	Me.			
%	100	50	100	2	10	50	100			

Fa Fb Fc Fc_1 Fc_2

F I G U R E 1 7

Two-dimensional TLC chromatogram of the $CHCl_3$-soluble portion from the ~~fraction of~~ $CHCl_3$-MeOH extract

The plate was developed in two solvents (ref. 49).

first solvent - $CHCl_3$-MeOH - ammonia (65:35:5 v/v)

second solvent - $CHCl_3$-acetone-MeOH-acetic acid H_2O (5:2:1:1:0.5)

O - origin

Sph - sphyngomyelin standard

GL - glycolipid from L. monocytogenes

DPG - beef heart cardiolipin standard

NL - neutral lipids

Fa, Fb, Fc_1 and Fc_2 - phospholipids in L. monocytogenes

F I G U R E 1 7

Two-dimensional TLC chromatogram of the $CHCl_3$-soluble portion from the $CHCl_3$-MeOH extract

The plate was developed in two solvents (ref. 49).

first solvent - $CHCl_3$-MeOH - ammonia (65:35:5 v/v)

second solvent - $CHCl_3$-acetone-MeOH-acetic acid H_2O (5:2:1:1:0.5)

O - origin

Sph - sphyngomyelin standard

GL - glycolipid from L. monocytogenes

DPG - beef heart cardiolipin standard

NL - neutral lipids

Fa, Fb, Fc_1 and Fc_2 - phospholipids in L. monocytogenes

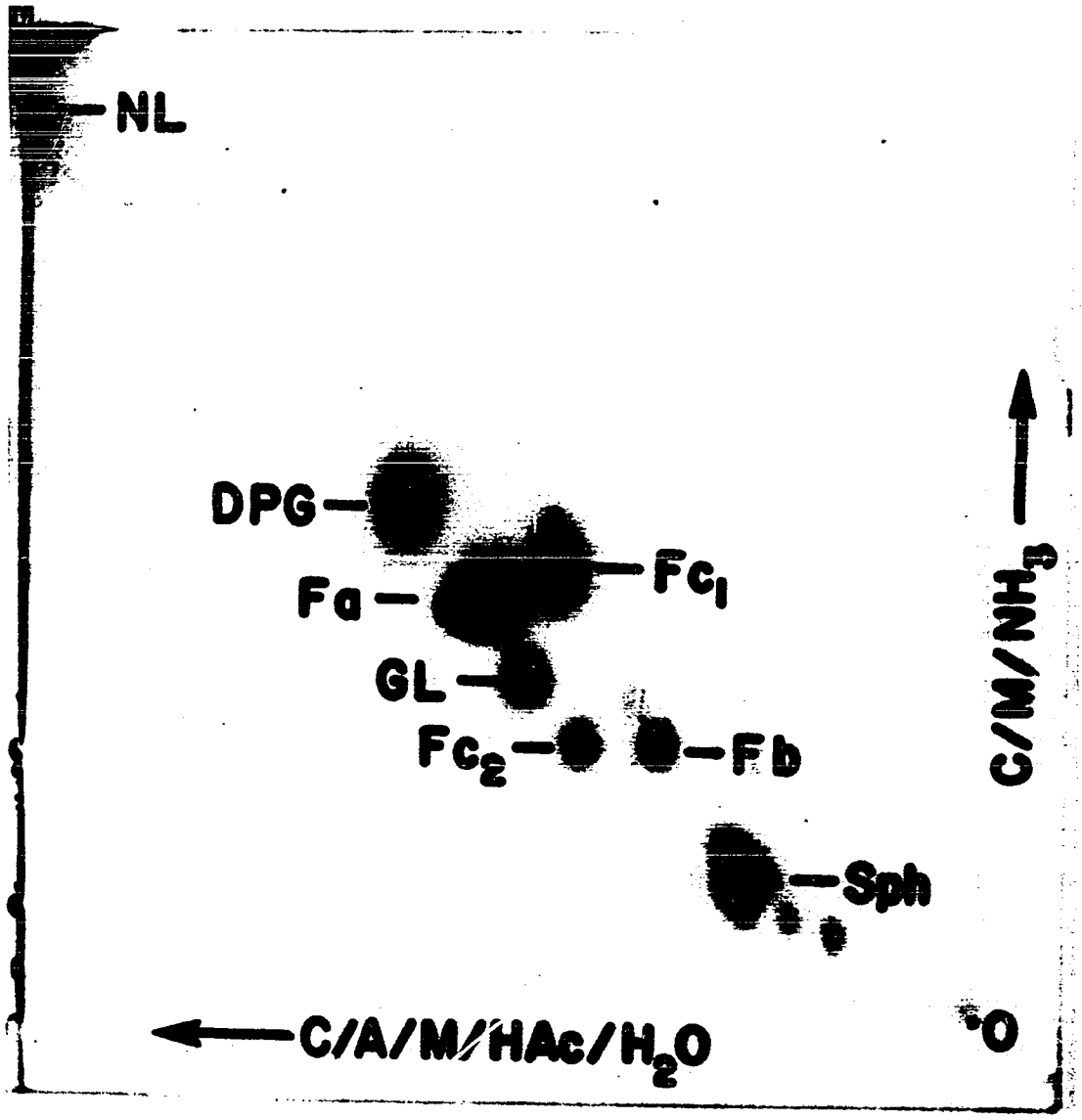

NL
DPG
Fa
Fc1
GL
Fc2
Fb
Sph
C/M/NH3
C/A/M/HAc/H2O
O

TABLE III

Relative proportions of phospholipids isolated from fraction F of the $CHCl_3$-soluble material

Isolated phospholipid	% of total phospholipids
Top spot (Fa)	45 - 50
Middle spot (Fb)	8 - 10
Bottom spot (Fc_1)	40 - 45
Bottom spot (Fc_2)	5 - 7

showed a positive reaction with the reagent for phosphatides (258) but they were all ninhydrin negative. Some ninhydrin positive material was, however, noticed in the area below the Fc spot on the plate developed in solvent A. The phospholipids were also insensitive to Dragendorf reagent as well as to ammoniacal silver nitrate reagent.

C. Chemical analysis

All isolated phospholipids were analyzed for fatty acid ester groups, phosphorus, glycol and glycerol. The results are represented in Table IV as molar ratios relative to phosphorus. It can be seen that the molar ratio of ester to phosphorus, glycol and glycerol in Fa is approximately 4:2:0:3. The ratios in Fb are 4:3:0:4 and in Fc_1 2:1:1:2, respectively. The ratio in Fc_2 is, however, 3:1:0.2:2. Whereas in the first three samples, no consumption of periodic acid was noticed, Fc_2 consumed some periodic acid during determination of glycol, which is represented by the 0.2 moles of glycol per mole of phosphorus.

D. Infrared spectroscopy

Infrared spectroscopy was also used to obtain information about the chemical structure of the isolated phospholipids (Figs. 18 and 19). Although the spectra are complicated and difficult to analyze, they give some information about the different groups in the molecules and an attempt was made to analyze the individual peaks by use of specific Tables and data reported in numerous publications. (54, 69, 215, 225, 237).

TABLE IV

Chemical analysis of intact phospholipids

Isolated phospholipid	Ester	Phosphorus	Glycol	Glycerol
Top spot (Fa)	4.20	2.00	0	2.80
Middle spot (Fb)	3.83	3.00	0	3.97
Bottom spot (Fc_1)	2.05	1.00	0.94	1.80
Bottom spot (Fc_2)	6.03	2.00	0.41	3.86

Molar ratios are shown in the Table. All results represent mean values of at least five determinations for phosphorus and fatty acid ester groups, and 8-10 determinations for glycol and glycerol, done at different time intervals, immediately after isolation of the corresponding purified material.

F I G U R E 1 8

Infrared spectra of the isolated phospholipids (Fa, Fb, Fc_1)

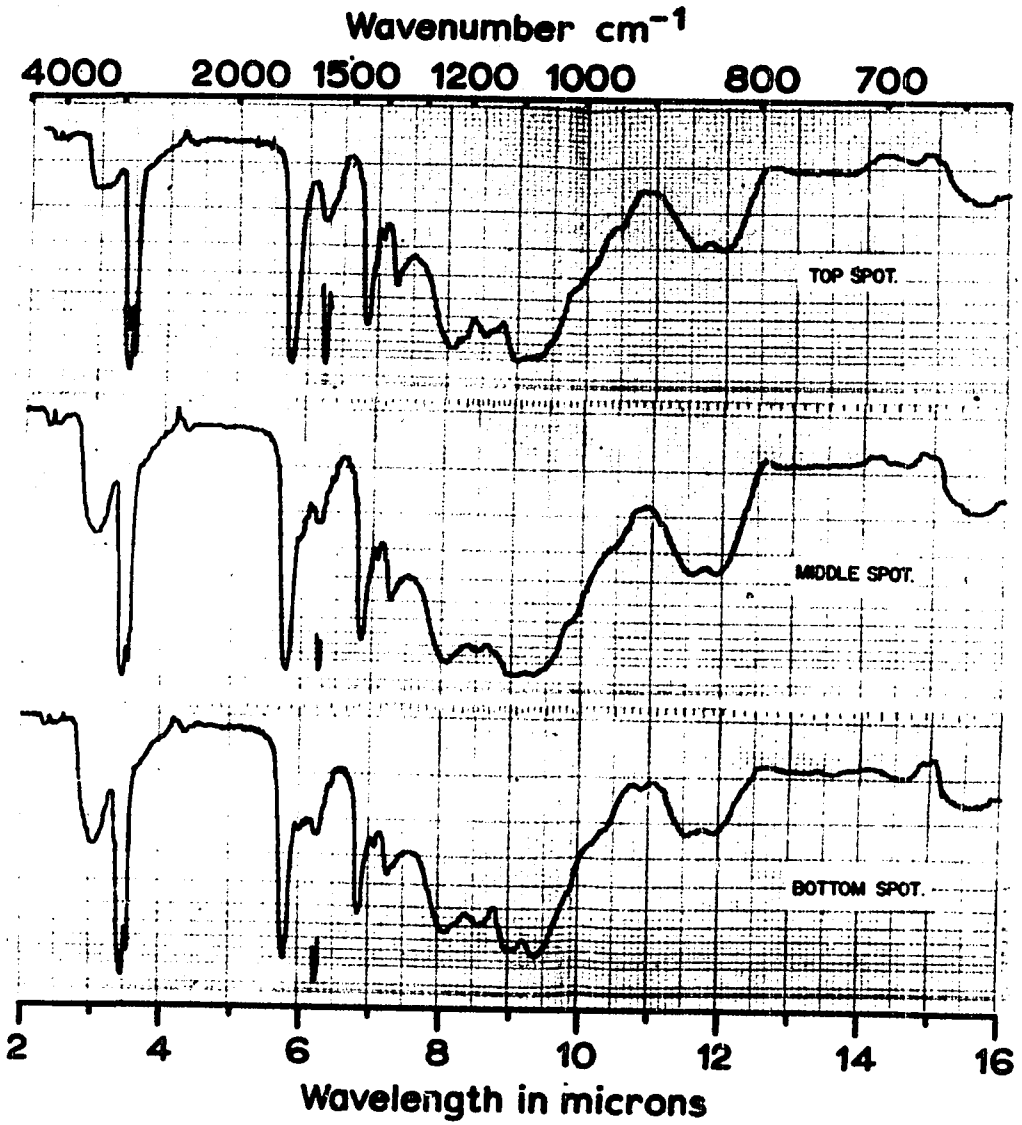
The spectra of the intact phospholipids from *L. monocytogenes* are recorded on a Beckman IR-10 Spectrophotometer.

top spot - Fa component

middle spot - Fb component

bottom spot - Fc_1 component

solvent - spectranalyzed $CHCl_3$


Wavenumber cm⁻¹
4000
2000
1500
1200
1000
800
700
TOP SPOT.
MIDDLE SPOT.
BOTTOM SPOT.
2
4
6
8
10
12
14
16
Wavelength in microns

F I G U R E 1 9

Infrared spectra of the isolated phospholipids (Fc_2, Fc_1)

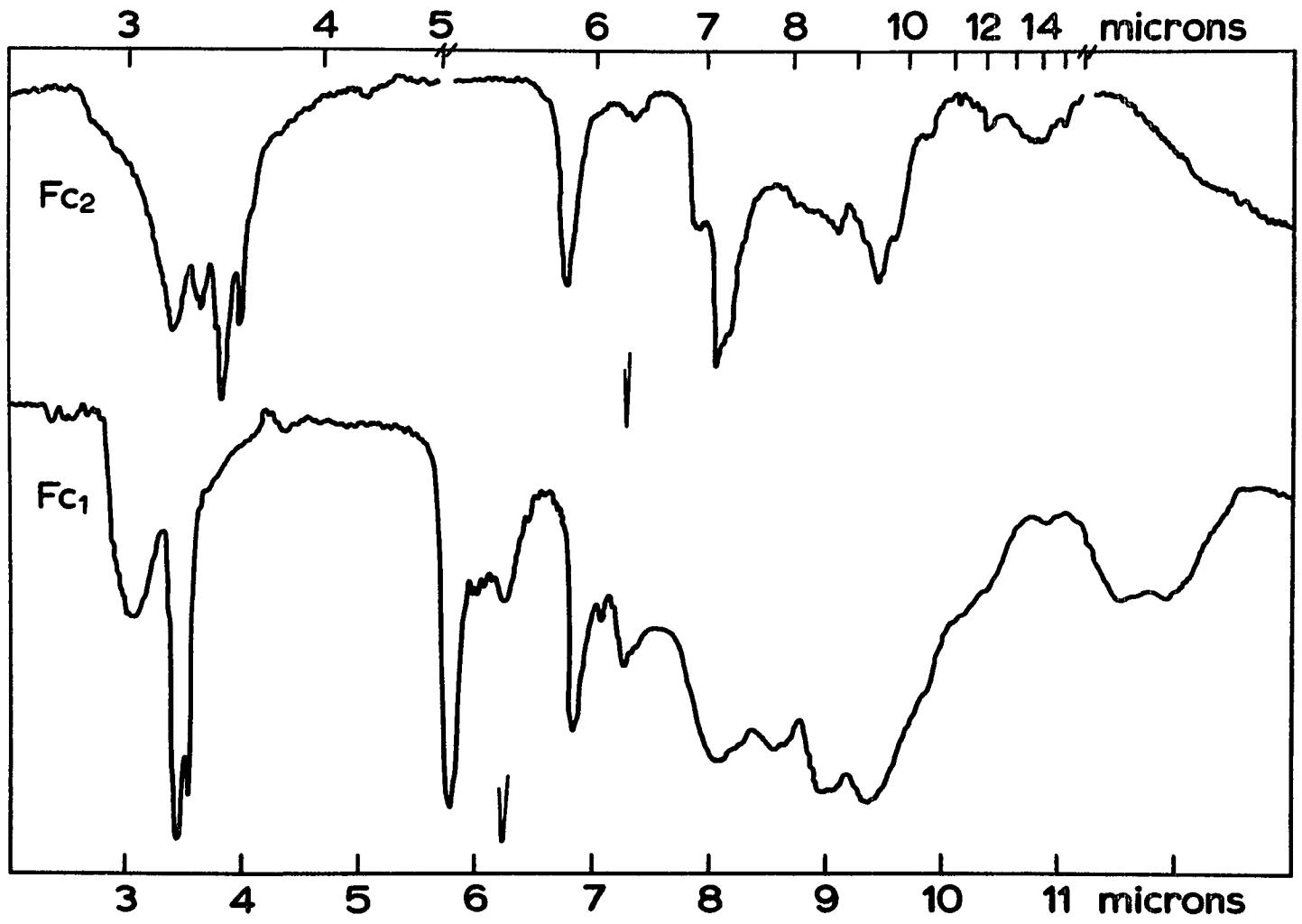
Spectra of intact Fc_2 and Fc_1 are recorded on IR-5A and IR-10 Beckman IR Spectrophotometers, respectively.

Fc_2 - separated bottom spot (lower R_f in solvent B)

Fc_1 - separated bottom spot (higher R_f in solvent B)

Solvent - spectranalyzed $CHCl_3$

IR Spectra of the original Fc_1 and Fc_2



The spectra of Fa, Fb and Fc_1 (top, middle and bottom) are presented in Figure 18. Comparing the three spectra, one can see that they are almost identical. There are minor differences, but the main peaks are very similar in all cases. This indicates that all three compounds have the same functional groups and seem to be closely related to one another. Analysing the spectra from higher to lower wavenumbers (5000 ——▬ 650), one can notice the first broad peak at 3320 cm^{-1} which is attributed to the -OH group, whereas the sharp peaks at 2980 and 2820 cm^{-1} are characteristic of -CH, $-CH_2$ and $-CH_3$ groups. The sharp peak at 1720 cm^{-1} is characteristic of an ester group (-COOR) and is present in all samples, while the peak at 1600 cm^{-1} can be ascribed to a carboxylic group (-C=O). The next peak at 1460 cm^{-1}, as well as the peak at 1370 cm^{-1}, is characteristic of $-CH_2-CH_3$ stretching vibrations. The latter could also be ascribed to a carboxylate. Peaks associated with the phosphate group could be the ones recorded at 1235 cm^{-1} (-P=O), 1065 cm^{-1} (-P-O-C-) and the small peak at 960 cm^{-1} could be due to the -P-OH group. The peak at 1165 cm^{-1} is probably due to an ester group (-COOR) whereas the broad peaks at lower wavenumbers (870 and 835 cm^{-1}) could be due to ethyl groups ($-CH_2-CH_3$).

The spectrum of Fc_2, as seen in Fig. 19, differs in many points from the other three, the spectrum of Fc_1 being presented for comparison. Although the two spectra were recorded on two different instruments, the wavelengths in microns are indicated on both spectra as well as the peak from the polystyrene standard (at 6.234) so that it is possible to compare them. As can be seen, the main differences

arise in the region of low wavelength (3-3.5 microns or 3330-2860 cm^{-1}). It is difficult to interpret these peaks, but they probably arise from -CH, $-CH_2$ and $-CH_3$ vibrations. Another major difference is seen in the region around 7 microns. The peak at about 6.9 microns is much smaller in Fc_2 spectrum and the peak near 7.1 microns larger than the corresponding peaks in Fc_1. Also, the fingerprint region from 7-12 microns shows considerable difference in Fc_2, whereas this region is very similar in the spectra of Fa, Fb and Fc_1. The series of peaks between 9 and 11 microns (1501 and 910 cm^{-1}) could possibly be attributed to -P-O-C- and -P-OH vibrations, although carboxylic groups can vibrate at these wavelengths.

III. PREPARATION AND ANALYSIS OF DEACYLATED PRODUCTS

A. Mild alkaline hydrolysis

The individual phospholipids and a cardiolipin standard were subjected to mild alkaline hydrolysis in dilute NaOH to split off the fatty acids and yield water-soluble deacylated products. These deacylated products were further purified and analyzed by different methods.

B. Anion-exchange and sephadex chromatography

Anion-exchange chromatography on Dowex 1-X2 was used to purify the individual deacylated products and this resulted in a clearcut separation and elimination of contaminants and artifacts, which were present in the material obtained after deacylation of the individual phospholipids. The results of this procedure are shown in several

Figures. Figs. 20 and 21 represent the results of phosphorus analysis in individual 10 ml samples collected during chromatography. Results with α-glycerophosphate standard (α-GP) and deacylated beef heart cardiolipin (GPGPG) are included. The elution pattern of other deacylated phospholipids such as glycerylphosphorylcholine (GPC), glycerylphosphorylethanolamine (GPE), glycerylphosphorylinositol (GPI) and glycerylphosphorylglycerol (GPG), according to Wells and Dittmer (268), is indicated.

The deacylated products from Fa and cardiolipin were eluted with the same strength of buffer, giving an indication that these compounds were identical. The deacylated middle spot was, however, eluted with a somewhat higher strength of buffer. Deacylated Fc_1 and Fc_2 (Fig. 21) were eluted together and at the place where GPG comes off the column, but these two compounds were later shown to be definitely different. The other minor peaks in the chromatograms are probably artifacts which came from degradation of the main components during extraction of the lipids, ATF column chromatography and mild alkaline hydrolysis. Evidence for such degradation was obtained by chromatographing a cardiolipin standard in comparison with the same sample, which was previously chromatographed on an ATF column (Fig. 22).

The deacylated products were effectively separated from these degradation products by the anion-exchange chromatography and pooled fractions containing the main components were used for further analytical studies.

The first step in this analysis was a lyophilization of the pooled fractions. It was hoped that this might also free the

F I G U R E 2 0

Anion-exchange chromatograms of deacylated top spot (Fa), middle spot (Fb) and standards (GP and GPGPG)

Elution patterns of Fa, Fb, α-GP and GPGPG are shown and the pattern for other deacylated phospholipids such as GPC, GPE, GPI and GPG is indicated according to Wells and Dittmer (268). Details in the text.

GPC - glycerylphosphorylcholine

GPE - glycerylphosphorylethanolamine

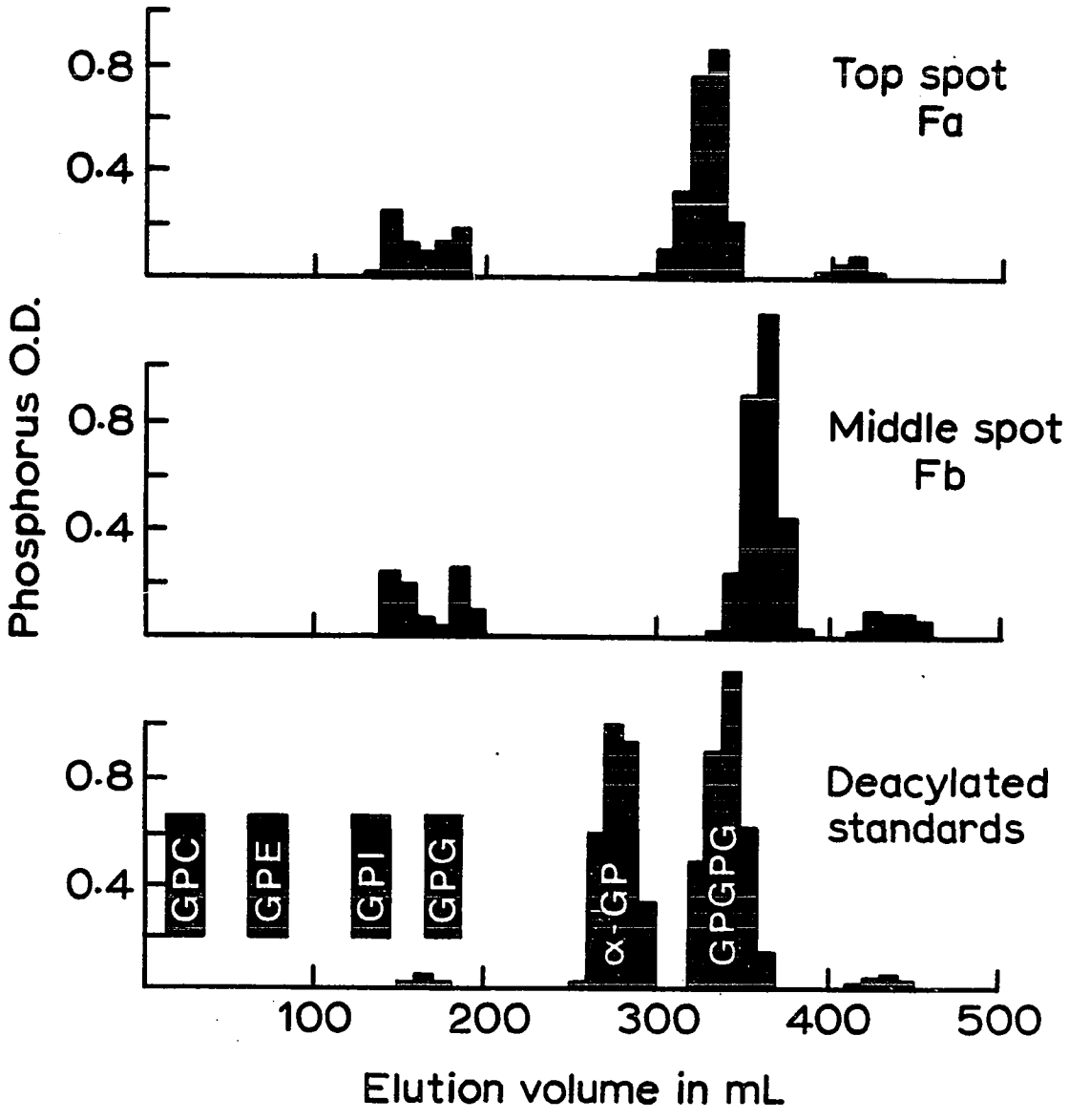
GPI - glycerylphosphorylinositol

GPG - glycerylphosphorylglycerol

α-GP - α-glycerophosphate

GPGPG - deacylated beef heart cardiolipin standard

Anion exchange chromatography
of deacylated products
of phospholipids from L.monocytogenes.



F I G U R E 2 1

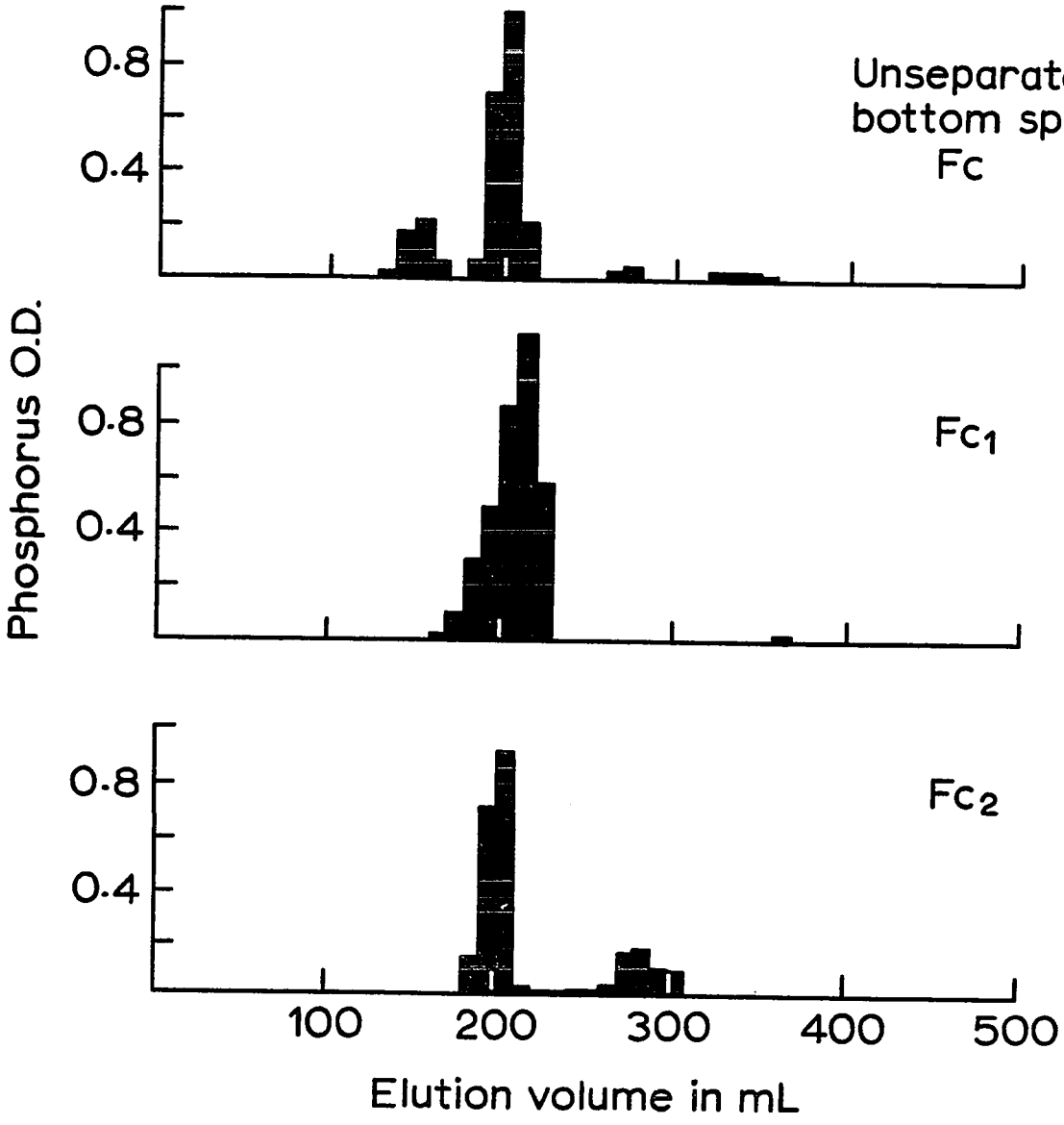
Anion-exchange chromatograms of deacylated non-separated and separated bottom spots

Fc - non-separated bottom spots

Fc_1 - bottom spot (higher R_f in solvent B)

Fc_2 - bottom spot (lower R_f in solvent B)

Anion exchange chromatography
of deacylated products
of phospholipids from L.monocytogenes.

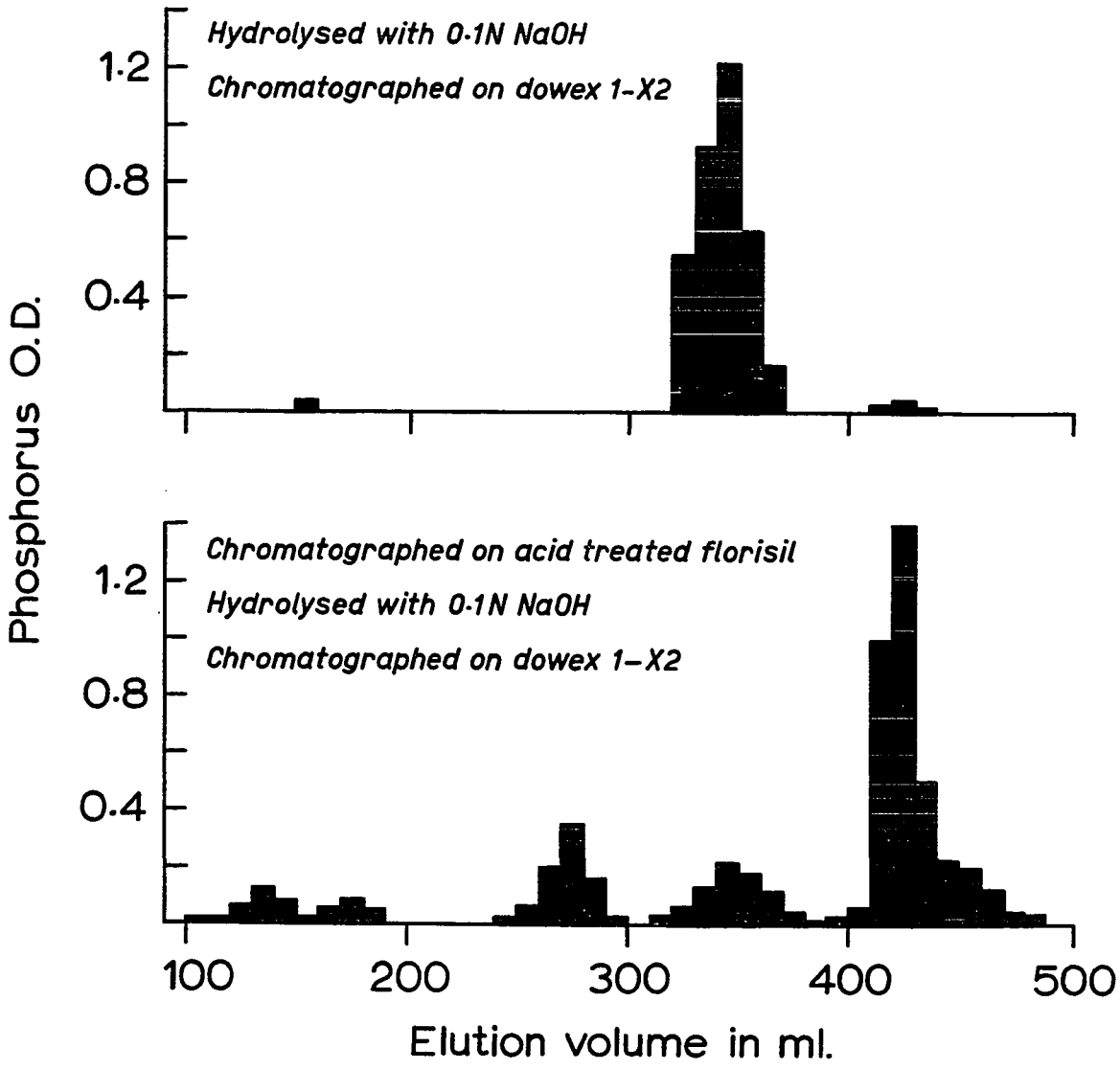


F I G U R E 2 2

Anion-exchange chromatograms of deacylated beef heart cardiolipin

A comparison of two chromatograms of differently treated cardiolipin standard are presented. Further details in the text.

ANION EXCHANGE CHROMOTOGRAPHY
OF DEACYLATED BEEF HEART CARDIOLIPIN



deacylated products from the accompanying salts which came from the formate-borate buffer and from the ions added during alkaline hydrolysis but this was not possible by this method. Attempts to extract the salts with different solvents were also unsuccessful. However, this goal was accomplished by column chromatography on Sephadex G-15 which resulted in complete elimination of the salts and recovery of pure phosphorus-containing material (Fig. 23). This material was further analyzed chemically as well as by paper chromatography and NMR spectroscopy.

C. Chemical analysis

The deacylated phospholipids, purified by anion-exchange and Sephadex chromatography, were analyzed for phosphorus and glycol groups. A deacylated beef heart cardiolipin standard was also analyzed and all results are represented as molar ratios in Table V.

D. Paper chromatography

The nature of deacylated phospholipids was also studied by paper chromatography in seven different solvent systems and the corresponding R_f values are represented in Table VI. A deacylated beef heart cardiolipin (B.H.Cl.) and α-glycerophosphate (α-GP) were also included in this analysis. It is clearly demonstrated by the R_f values that none of the four investigated compounds had the same chromatographic characteristics in all solvent systems used. This difference in R_f values indicated that none of the compounds examined had the same "molecular backbone", i.e., the possibility of lyso

F I G U R E 2 3

Purification of water soluble hydrolysis products on Sephadex G-15 column

The phosphorus containing peak is presented and the elution pattern of accompanying ions is indicated. Further details in the text.

PURIFICATION OF WATER SOLUBLE HYDROLYSIS PRODUCTS ON SEPHADEX G15 COLUMN.

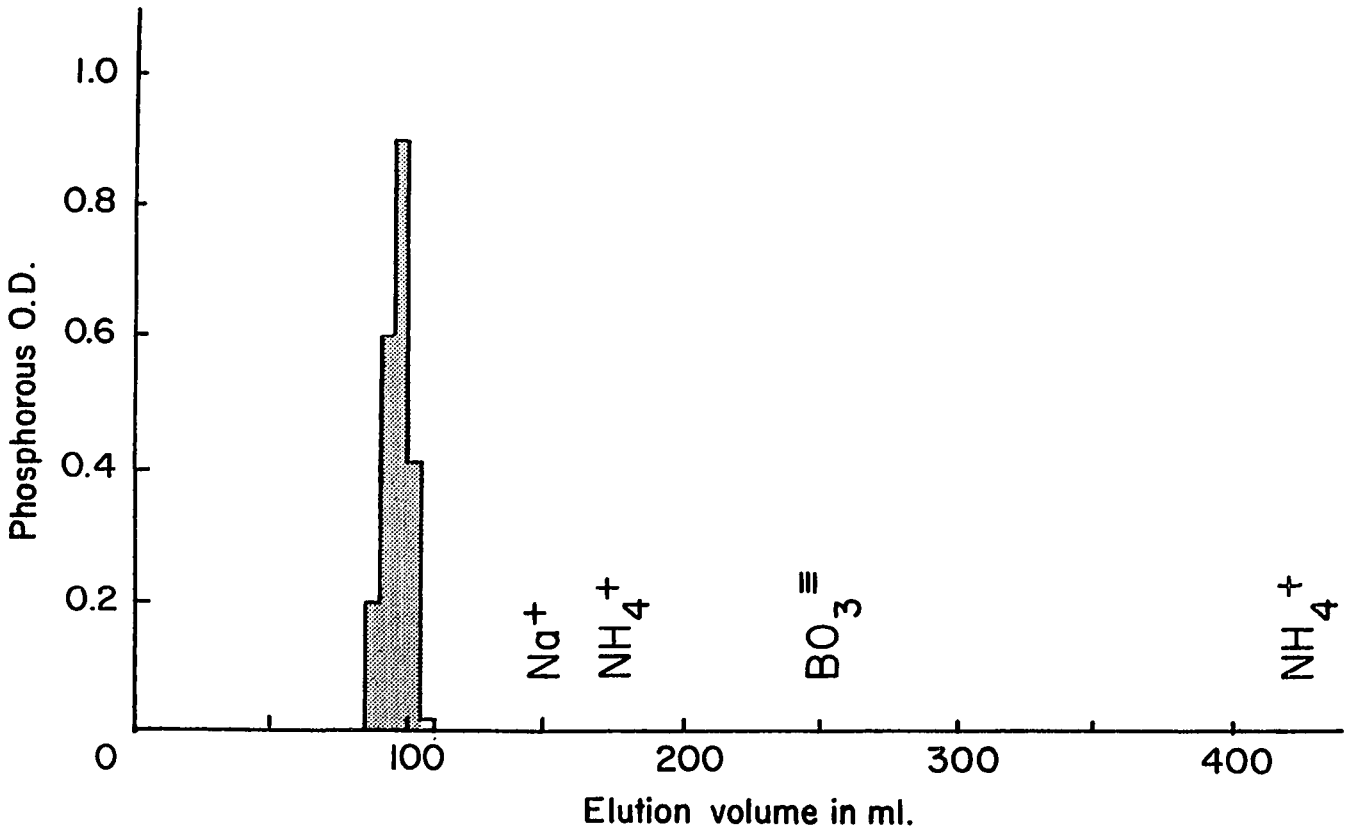


TABLE V

Chemical analysis of the deacylated phospholipids

Deacylated phospholipid	Phosphorus : Glycol	
Beef heart cardiolipin	1.00	0.97
Top spot (Fa)	1.00	1.00
Middle spot (Fb)	3.00	1.99
Bottom spot (Fc_1)	1.00	1.98
Bottom spot (Fc_2)	2.00	3.43

Results are represented as molar ratios relative to phosphorus. The groups were analyzed as shown in Table IV.

TABLE VI

R_f values obtained by paper chromatography of the deacylated phospholipids

Fa, Fb, Fc_1 and Fc_2 - the corresponding deacylated phospholipids

B.H.Cl. - beef heart cardiolipin standard

α-GP - α-glycerophosphate standard

DEACYLATED PHOSPHOLIPIDS OF LISTERIA MONOCYTOGENES.

PAPER CHROMATOGRAPHY—R_f VALUES.

SOLVENT SYSTEM	ISOLATED PHOSPHOLIPID				STANDARDS	
	Fa	Fb	Fc_1	Fc_2	B.H.CL.	∝-GP
Isopropanol–Ammonia–Water (7:1:2)	0.16	0.23	0.43	0.18	0.17	--
T.But.–Water–Trichlorooc. acid(62:38:10%)	0.58	0.28	0.40	0.16	0.59	—
Phenol saturated with 1% Ammonia	0.24	0.13	0.43	—	0.24	—
Butanol–Propionic acid–Water (5:7:10)	0.58	0.54	0.57	—	0.58	—
Propanol–Ammonia–Water (6:3:1)	0.22	0.26	0.45	0.22	0.22	—
Butanol–Acetic acid–Water (5:4:1)	—	—	0.21	0.03	—	0.5
1 M. Am. acetate – Abs. Ethanol(35:65)	—	—	0.78	0.74	—	0.51

compounds and compounds which were originally esterified with fatty acids at different places in the molecule was excluded. The separation between individual spots was in many cases satisfactory but the best separation was obtained in the tert-butanol-water-trichloroacetic acid system, and a good separation was also obtained with isopropanol-ammonia-water.

The R_f values of deacylated Fa and B.H.Cl. were identical in all solvents used (first 5 solvents) and this was in agreement with other findings which all give strong support for the identity of these two compounds. There is, however, considerable difference between Fc_1 and Fc_2 samples which were well-separated in all solvents tested.

E. Nuclear magnetic resonance spectroscopy

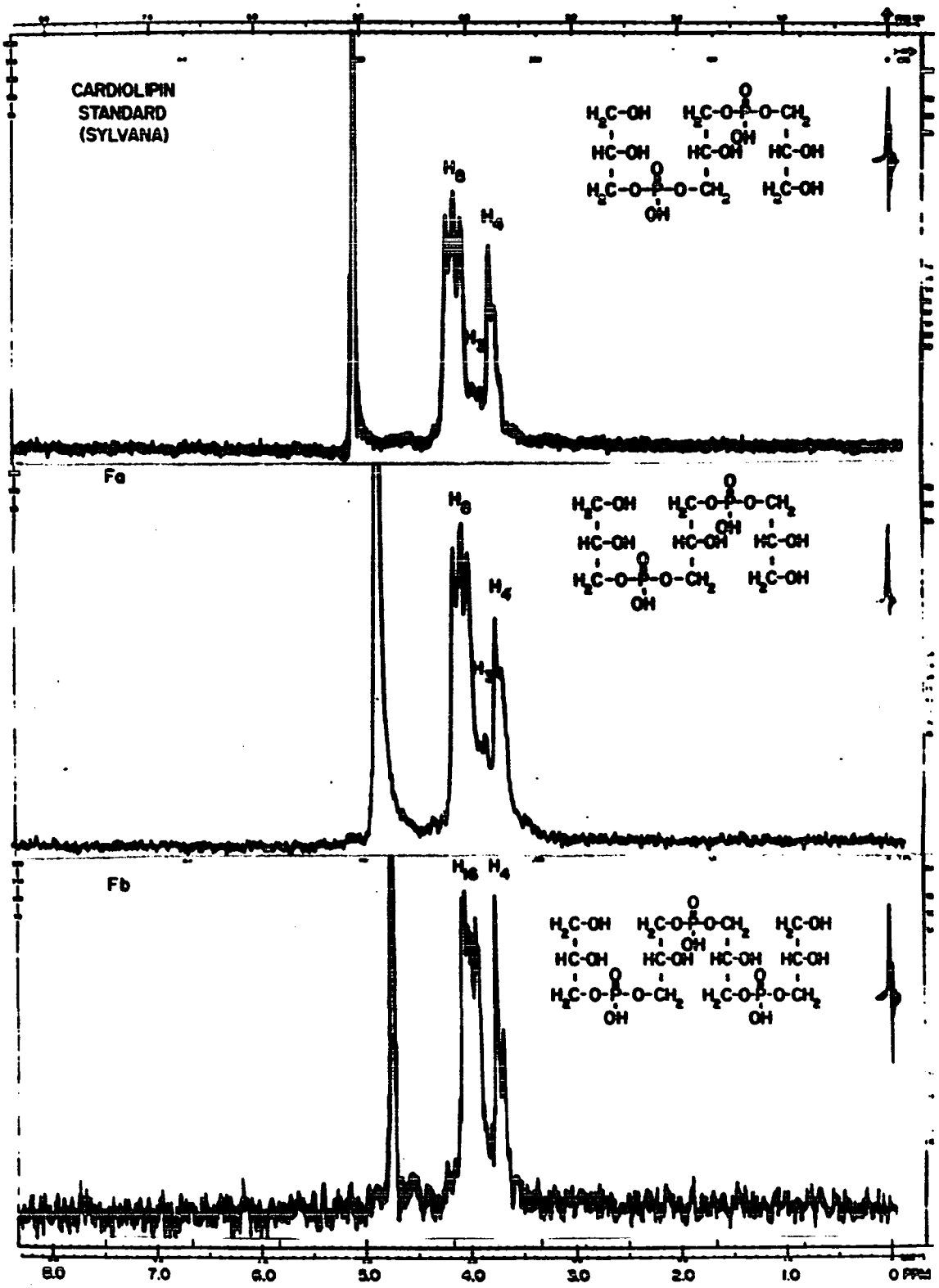
Valuable information about the structure of the phospholipids was obtained by NMR spectroscopy of their deacylated products. The spectra of three compounds (deacylated cardiolipin standard, Fa and Fb) are shown in Fig. 24. The identity of the first two spectra (cardiolipin and Fa) can be easily recognized and the spectra are compatible with the bis-glycerylphosphorylglycerol structure (GPGPG) which has been assigned to the deacylated product of beef heart cardiolipin. The four protons resonating at the highest field are thought to be the protons on position 1 of the terminal glycerols, as they are approximately equivalent and the deshielding effect of the high electron density phosphate group is minimal. The three protons on the 2- position of the three glycerols are more strongly influenced by the phosphate group and they are expected to resonate at a somewhat

F I G U R E 2 4

Nuclear magnetic resonance spectra of the deacylated cardiolipin standard, Fa and Fb

The spectra were recorded on an A-60 NMR spectrometer. The resonance of the external standard (TMS) is shown in the right upper corner of the spectra. The corresponding protons are indicated above the peaks. The samples were dissolved in D_2O.

Spectroscopic variables	Cardiolipin	Fa	Fb
sweep width (CPS)	500	500	500
sweep time (SEC)	500	500	500
filt. band (CPS)	2	2	0.4
R. F. field (MG)	0.2	0.2	0.2
spec. amp.	32	50	200


CARDIOLIPIN
STANDARD
(SYLVANA)
Fa
Fb
H_B
H_A
H_3
H_4
H_{1B}
6.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0 PPM

lower field. However, these three protons are not expected to be approximately equivalent as the overall environment of the hydrogen atoms on the central glycerol differs from that of the terminal ones. Therefore, considerable overlapping of these peaks with the others is to be expected. The other 8 protons, which are located on the carbons esterified to the phosphate, are expected to resonate at the lowest field as the effect of vicinal phosphate on their resonance is considerable. The chemical shifts of the two major peaks in these two spectra are the same, as well as the areas under the peaks, which correspond to the theoretical for such a compound. The sharp peak at ca 4.8 ppm represents the hydrogens from the -OH groups as well as the ones from H_2O present in the D_2O used to dissolve the NMR samples.

The third spectrum in the Figure differs slightly from the two above in chemical shift as well as in the area under the peaks. A proposed structure, based on this spectrum and on the chemical analysis is shown on the chart. The peak resonating at the higher field is again attributed to the four hydrogen atoms on the 1-carbon of glycerol, whereas all other hydrogens (except the ones on -OH) are expected to resonate at a lower field and are, therefore, represented by the broad peak appearing at the lower energy.

The spectra in Fig. 25 represent an α-glycerophosphate standard (α-GP) together with deacylated Fc_1 and Fc_2. Deacylated Fc_1 was thought to be glycerylphosphorylglycerol (GPG) on the basis of chemical analysis and chromatographic behaviour, and the NMR spectrum is in agreement with this idea. As expected, the 4 protons on the 1 positions of the glycerols resonate at a higher field than the other 6 protons.

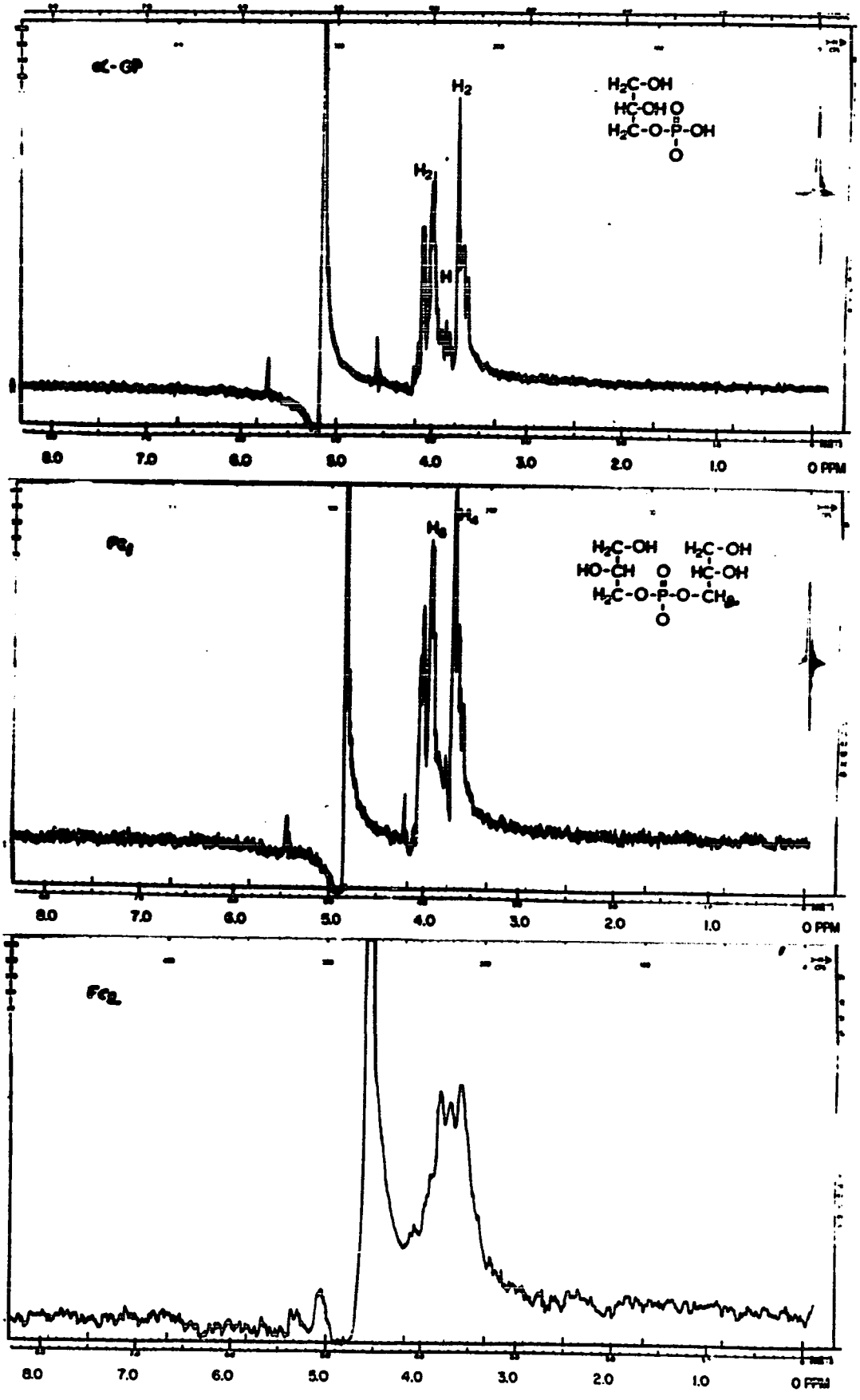
F I G U R E 2 5

Nuclear magnetic resonance spectra of

α-glycerophosphate and the deacylated Fc_1 and Fc_2

The spectra were recorded on an A-60 NMR spectrometer with external TMS standard. The solvent used is D_2O. The corresponding protons are indicated above the peaks.

Spectroscopic variables		α-GP	Fc_1	Fc_2
sweep width	(CPS)	500	500	500
sweep time	(SEC)	500	500	500
filt. band	(CPS)	2	1	0.1
R. F. field	(MG)	0.2	0.2	0.2
spec. amp.		20	50	200


α-GP
H2
H2
H
H2C-OH
HC-OH O
H2C-O-P-OH
O
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0 PPM
H6
H2C-OH H2C-OH
HO-CH O HC-OH
H2C-O-P-O-CH
O
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0 PPM
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0 PPM

The similarity between this spectrum and that of α-glycerophosphate may also be noted.

The spectrum of deacylated Fc_2, however, is seen to differ from the others. Although the resonance occurs in the same area of the spectrum, which could indicate a deacylated phospholipid molecule, the spectrum is not well resolved. A better resolution was obtained with an HA-100 NMR-spectrometer as shown in Fig. 26. In this case, three main peaks can be clearly observed, and in addition there are two minor doublets appearing at the lowest field. To analyze such a complex spectrum fully would be very difficult and only an approximate interpretation is presented here. Again, the protons resonating at the highest field would be the ones most remote from the phosphate groups, whereas the peaks at the lowest field represent the protons which are under the greatest influence of these groups.

One can definitely say that, according to the NMR spectra, the deacylated phospholipids Fa, Fb and Fc_1 are homologous in the structural arrangement of their phosphate and glycerol moieties, whereas the component Fc_2 obviously differs but still belongs to the same group of compounds.

IV. PREPARATION AND ANALYSIS OF PRODUCTS OF ENZYMATIC DEGRADATION

A. Enzymatic degradation

The treatment of isolated phospholipids with various phospholipases was included in this investigation primarily in order to get information about the distribution of fatty acids in the various phospholipid molecules. It is well-established that phospholipase A

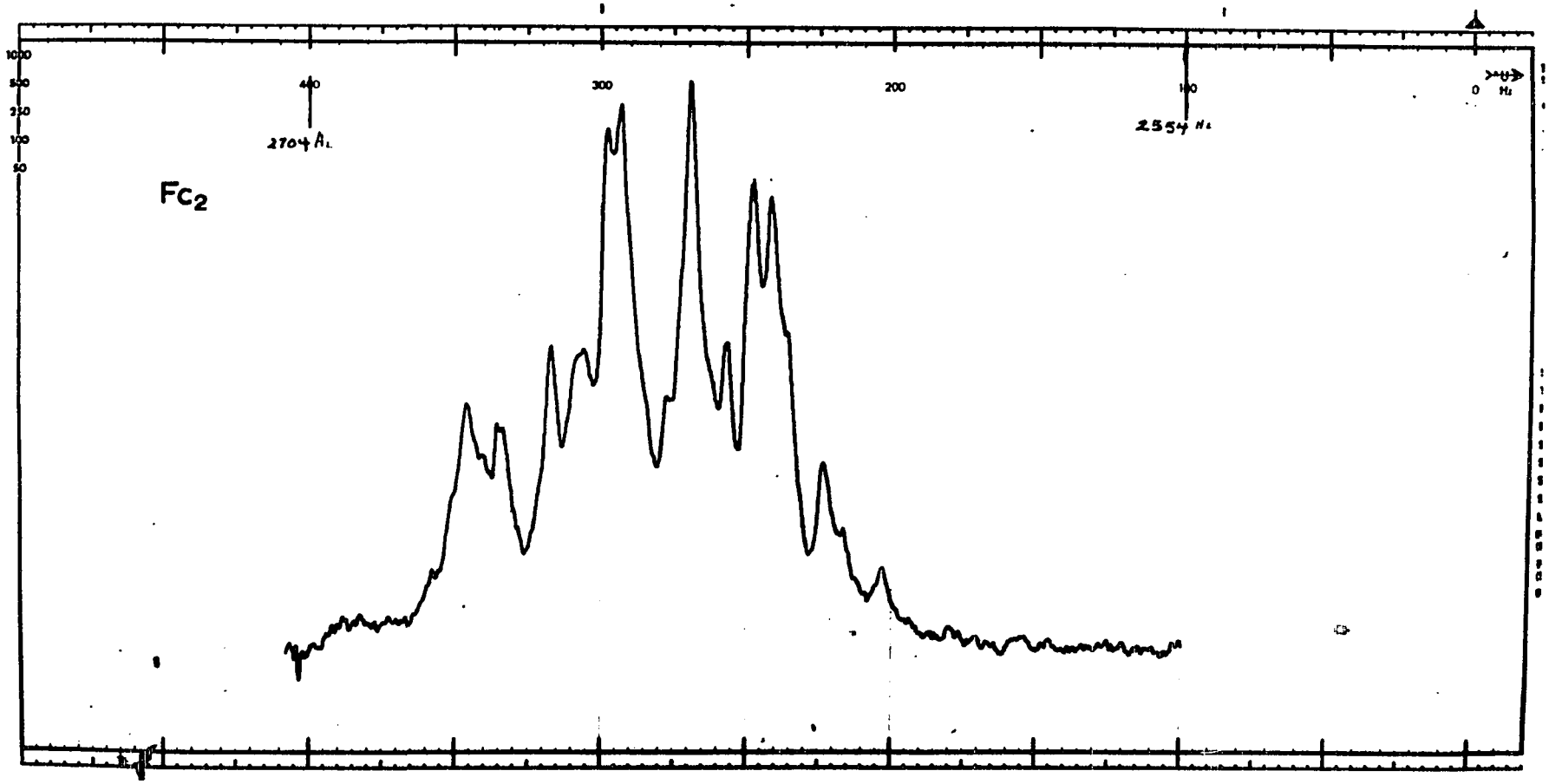
F I G U R E 2 6

Nuclear magnetic resonance spectrum of deacylated Fc_2

The spectrum was recorded on a H-100 NMR spectrometer.

Frequency of the applied field is presented in Hz.

solvent	- D_2O
temp.	- 32°C
freq. response	- 0.2 Hz
R. F. attenuator	- 29 db
sweep time	- 1000 SEC
sweep width	- 250 Hz
sweep offset	- 769 Hz
spectrum amp.	- 8000
lock signal	- 2 V
man. osc. freq.	- 0.3 m. gauss
sweep freq.	- 0.5 m. gauss
field sweep	- yes


Fc2
2704 Hz
2554 Hz
400
300
200
100
0
Hz

from snake venom preferentially liberates the fatty acid esterified to the β-position, and analysing the rest of the molecule for fatty acids as well, one can obtain information on whether the acids are randomly or non-randomly distributed in the molecule.

The mixture resulting from treatment of a phospholipid with phospholipase A is composed of the liberated fatty acids, one or more lyso derivatives of the original compound, and any material not hydrolyzed during the procedure. In our experiments with a collidine-buffered system developed by Magee and Thompson (180) and using an activated and partially purified enzyme from snake venom, the phospholipids were almost completely split into their lyso derivatives and the free fatty acids. These two main products could be easily separated from one another, either by preparative TLC or by column chromatography on ATF using $CHCl_3$ to elute the fatty acids and $CHCl_3$-MeOH (1:1) to elute the lyso derivatives. The results can be seen in Figs. 27, 28 and 29. In Fig. 27, three plates are shown, the top one representing the mixture of products obtained. Fa, Fb and Fc_1 represent the product mixtures from the corresponding starting material ST Fa, ST Fb and ST Fc_1. Free C_{15} and C_{17} anteiso acids are included as standards. The plate was developed in solvent A. The lower two plates, developed in different solvents, as indicated, show the same products after separation on an ATF column. Lyso derivatives are represented by L, and the corresponding original phospholipids by ST.

The results obtained after treatment of Fc_2 with phospholipase A are represented in Fig. 28. The isolated Fc_2 is represented by ST, whereas S represents the resulting mixture after treatment, and C_{15}

FIGURE 27

TLC chromatograms of the products obtained after treatment of Fa, Fb and Fc_1 with phospholipase A

The top chromatogram was developed in solvent A as well as the left bottom one. The right bottom chromatogram was developed in solvent C.

solvent A - $CHCl_3$-MeOH-H_2O (65:20:3)

solvent C - petr. ether-ether-acetic acid (60:40:1)

Fa, Fb, Fc_1 - products obtained after treatment of ST-Fa, ST-Fb and ST-Fc_1 respectively.

FA - fatty acid standards

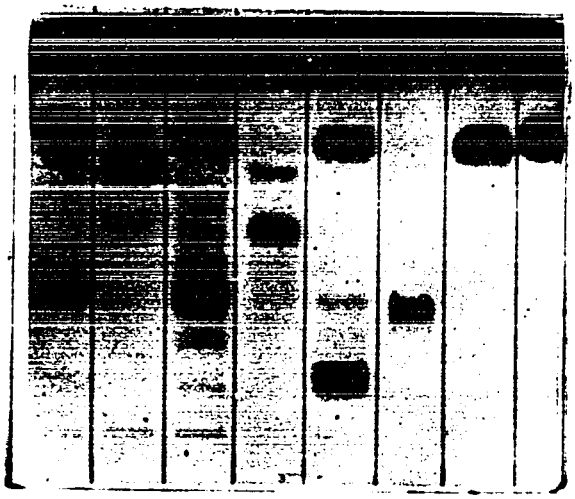
C_{15}, C_{17} - C_{15} and C_{17} anteiso acids

L - lyso compound

ST - standard

TREATMENT WITH PHOSPHOLIPASE A

PRODUCTS

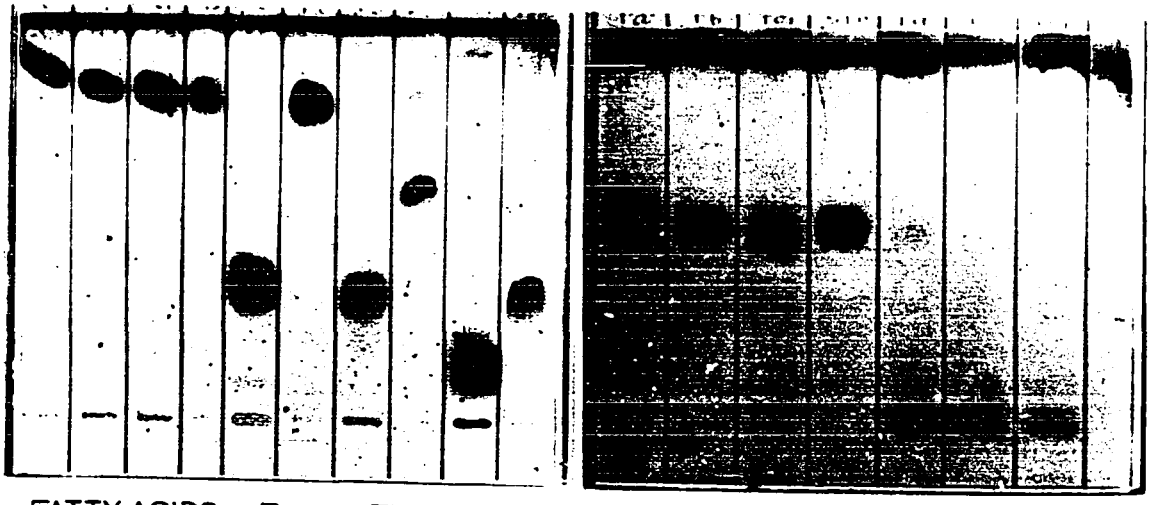


Fa ST Fa Fb ST Fb Fc_1 ST Fc_1 FA C_{15} C_{17}

SEPARATED PRODUCTS

C-M-W

PET-ET-HAC



FATTY ACIDS Fa Fb Fa_1 ST L ST L ST L ST

Fa Fb Fc_1

FATTY ACIDS Fa Fb Fc_1 ST

LYSO Fa Fb Fc_1

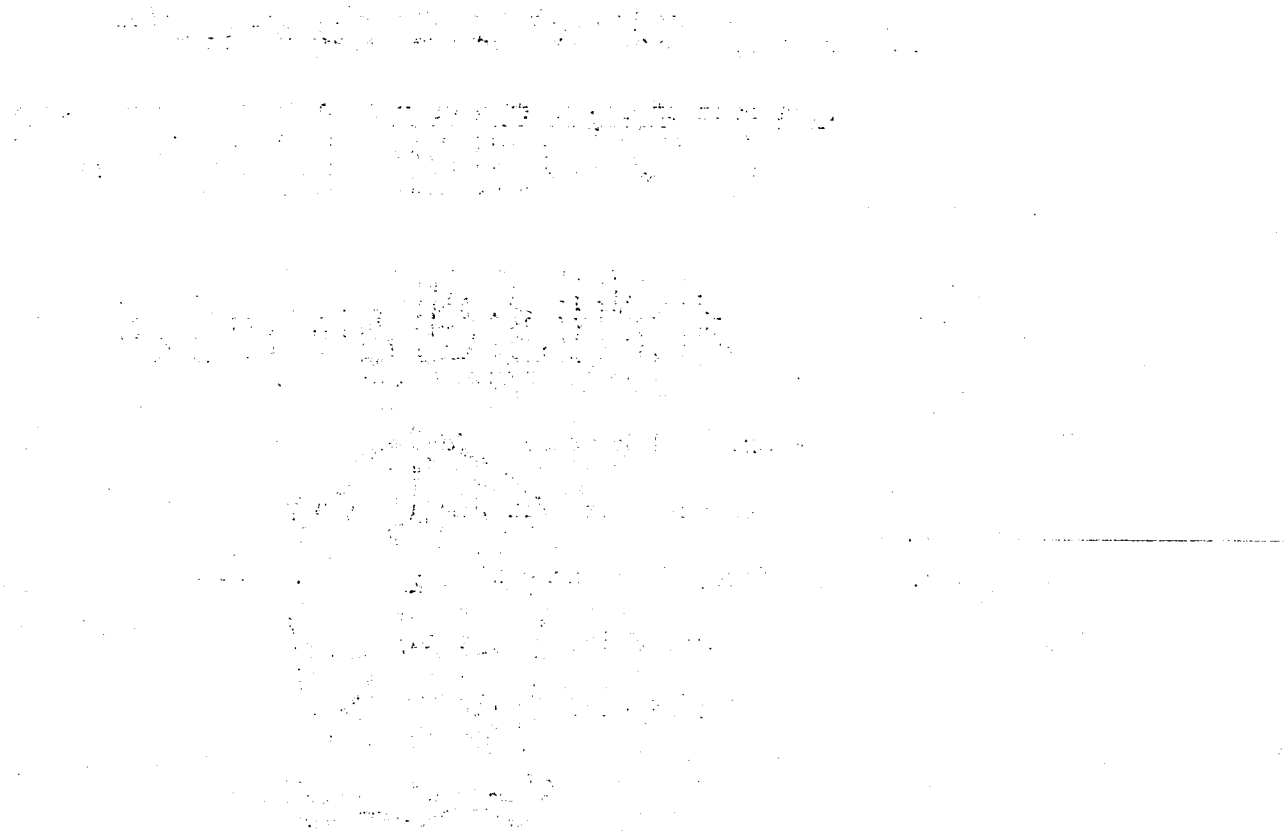


FIGURE 28

TLC chromatograms of the products obtained after treatment of Fc_2 with phospholipase A

C-M-W - $CHCl_3$-MeOH-H_2O (65:20:3)

C-M-AM - $CHCl_3$-MeOH-28% ammonia (65:35:5)

ST - original untreated Fc_2

S - products after treatment with phospholipase A

FA - fatty acid standard (C_{15} anteiso acid)

L - separated lyso compound

C_{15} - acids liberated with phospholipase A

TREATMENT OF FC_2 WITH PHOSPHOLIPASE A

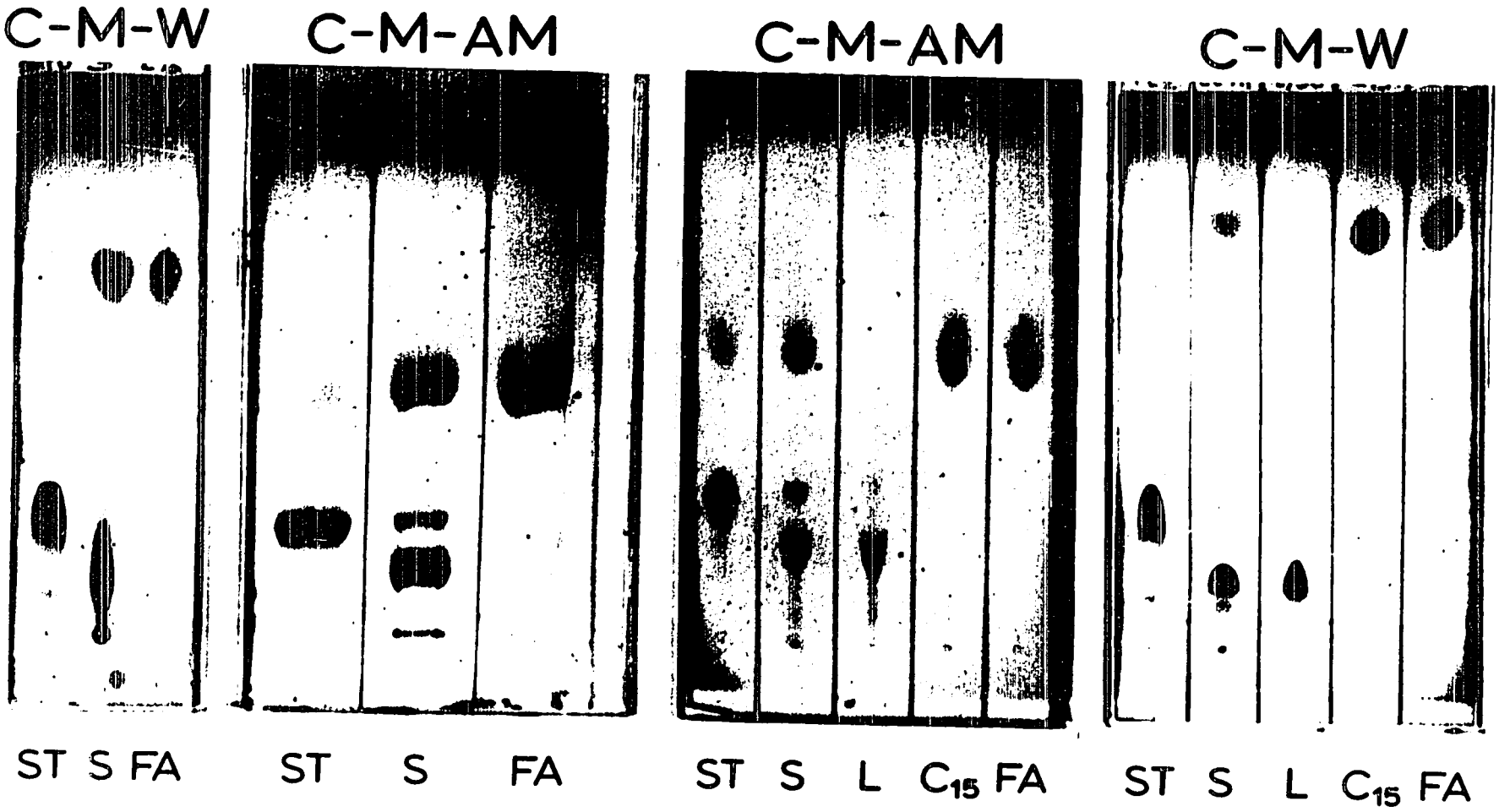


FIGURE 29

TLC chromatograms of the lyso derivatives obtained after treatment of Fa, Fb, Fc_1 and Fc_2 with phospholipase A

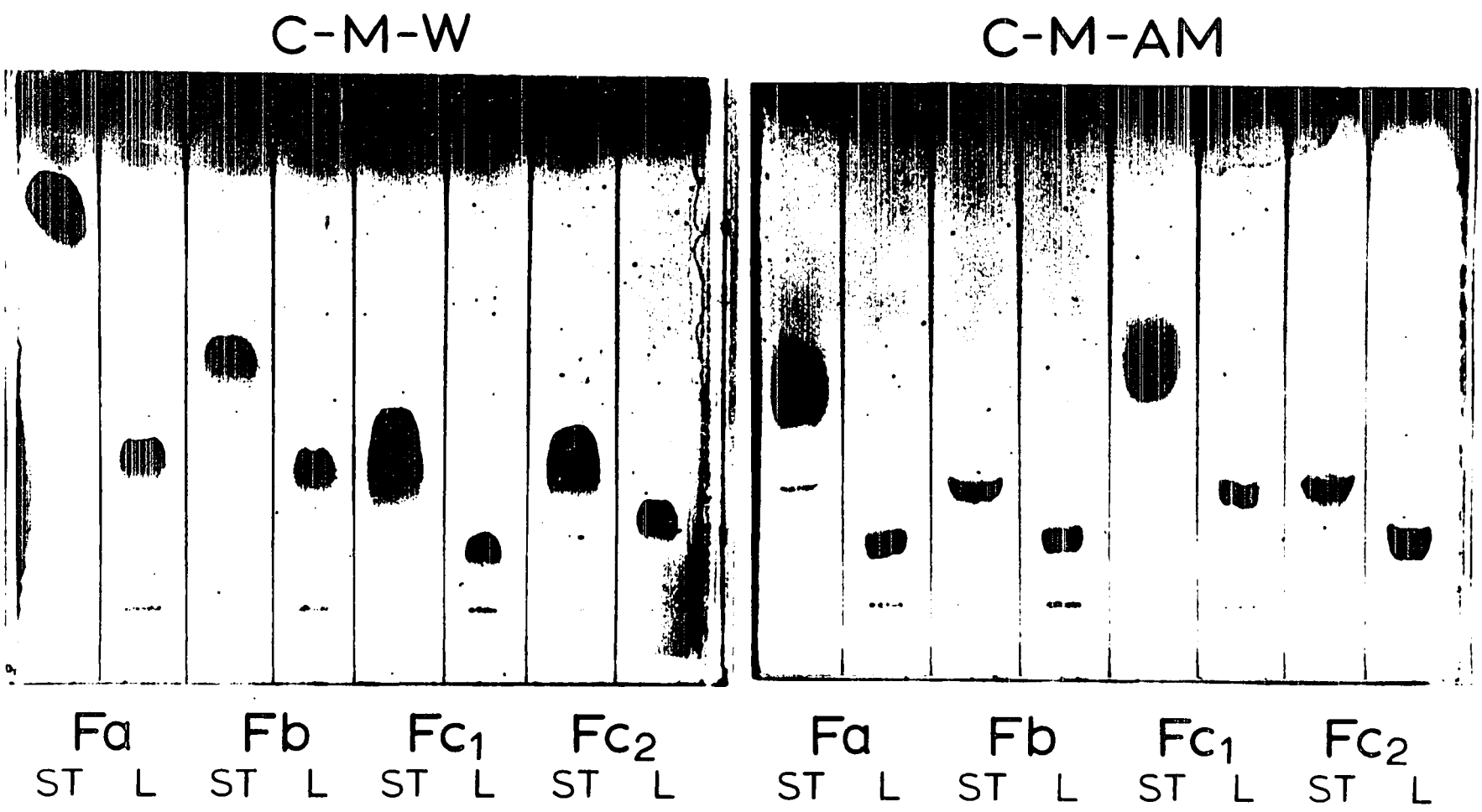
C-M-W - $CHCl_3$-MeOH-H_2O (65:20:3)

C-M-AM - $CHCl_3$-MeOH-28% ammonia (65:35:5)

ST - untreated phospholipid

L - lyso derivative obtained

TREATMENT WITH PHOSPHOLIPASE A



the separated fatty acid. One can also see here that the degradation with the enzyme was almost complete, although some original material was still present after treatment for 48 hours, as seen in sample (S) on the second chromatogram from the left. This material was, however, separated from the lyso compound (L), as demonstrated in other chromatograms.

A summary of the results for all four phospholipids with the two solvent systems is represented in Fig. 29. It can be seen that the lyso derivatives had smaller R_f values than the original compounds in all cases, as expected. The original and lyso compounds had in some cases similar R_f values, but further analyses showed them to be different compounds in each case.

Attempts were made to degrade the corresponding phospholipids with phospholipases C and D as well, but in nearly all cases, these attempts were unsuccessful, even after prolonged contact of the enzyme with the substrate. However, a positive result was obtained when Fc_1 was used as substrate for phospholipase C from Cl. welchii. About 60% hydrolysis was achieved and 1, 2-diglyceride was liberated, as demonstrated in Fig. 30.

B. Chemical analysis

The lyso compounds isolated from the reaction mixture after treatment of the corresponding phospholipids with phospholipase A were all analyzed for phosphorus, glycol and fatty acid ester groups. The results, representing molar ratios relative to phosphorus are shown in Table VII.

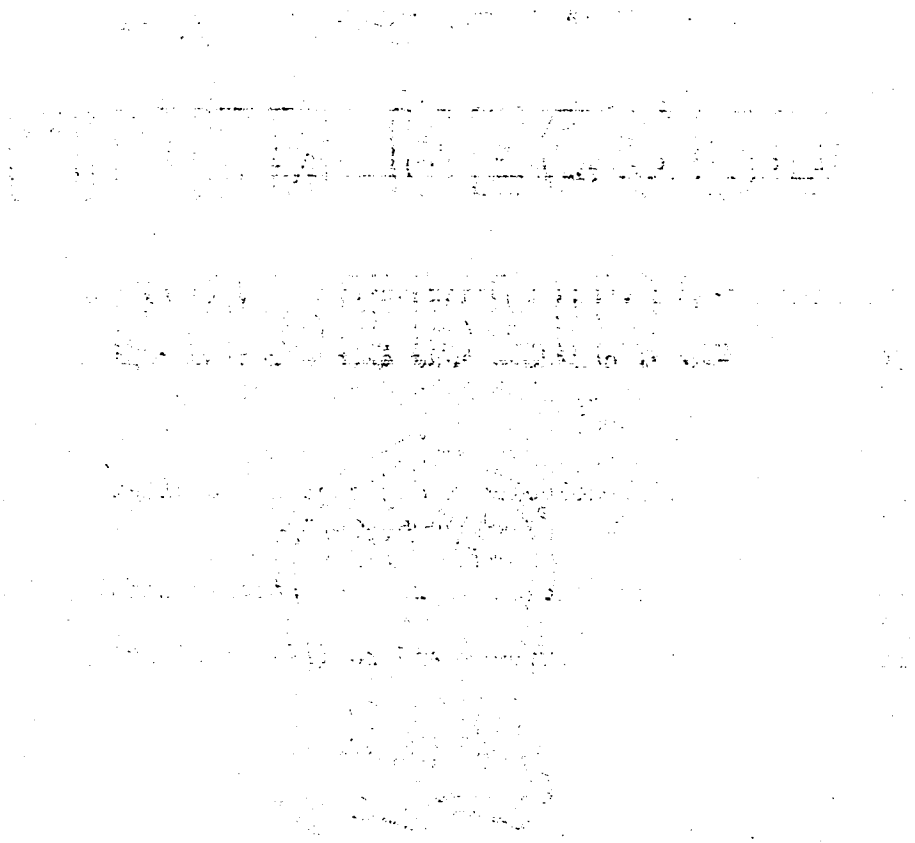


FIGURE 30

TLC chromatogram of the products obtained after treatment of Fc_1 with phospholipase C

PET-ET-HAC - petr. ether-ether-acetic acid (60:40:1)

DG - diglyceride standard (the top spot represents 1, 3-DG and the bottom spot 1, 2-DG)

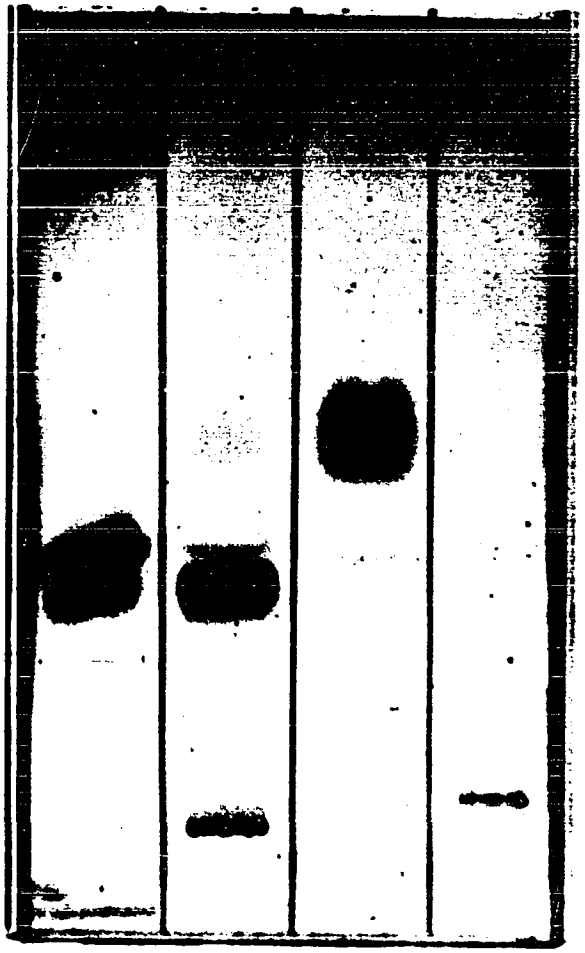
S - $CHCl_3$-soluble products obtained after treatment with phospholipase C

FA - fatty acid standard (C_{15} anteiso acid)

MG - monoglyceride standard (1-monopalmitin)

TREATMENT OF FC_1 WITH PHOSPHOLIPASE C

PET-ET-HAC



DG S FA MG

The ratios obtained by analysing the lyso derivatives of Fa, Fb and Fc_1 are in agreement with the results expected for the isolated phospholipids without the fatty acids on the β-position and give further support for the proposed structures of the three compounds. In this way, the Fa component represents a lyso diphosphatidylglycerol lacking two fatty acids present in the original compound. Two fatty acids are also lacking in the lyso Fb, corresponding to a lyso-bis-diphosphatidylglyceryl phosphate structure. The lyso Fc_1 corresponds to a lyso phosphatidylglycerol with a free glycol group and one fatty acid.

Lyso Fc_2 contained 2.5 ester groups and 0.65 moles of glycol per mole of phosphorus. These molar ratios are difficult to interpret as they do not represent full numbers or numbers near unity, and, therefore, the values were multiplied by 2, which gave more reasonable ratios for interpretation. According to this, there are 5 fatty acid ester groups in this lyso derivative which originated from the phospholipid having 6 ester groups (Table IV). The splitting of one fatty acid from the original molecule also resulted in formation of approximately one glycol group per two moles of phosphorus. Results obtained by other methods also showed a marked difference between the first three phospholipids and the Fc_2 component.

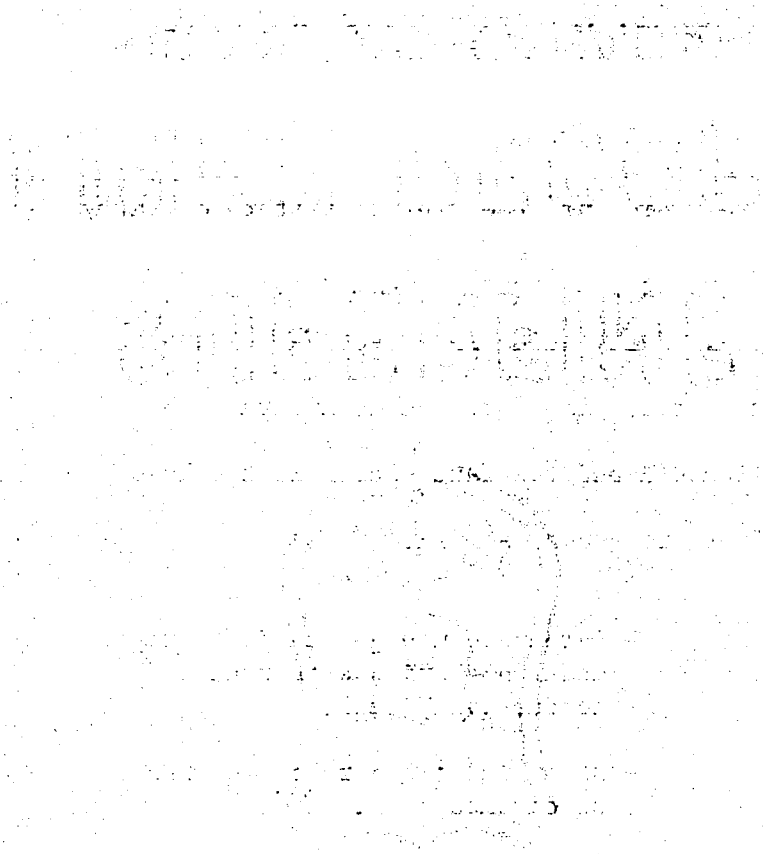
C. Gas-liquid chromatography of the fatty acids

The fatty acid composition of the individual phospholipids isolated from Listeria monocytogenes is represented in Figs. 31 and 32 and in Table VIII. The main fatty acids found in these compounds

TABLE VII

Chemical analysis of lyso compounds obtained by treatment of phospholipids with phospholipase A

Lyso compound from:	Ester	Phosphorus	Glycol
Top spot (Fa)	2.20	2.00	0
Middle spot (Fb)	1.94	3.00	0
Bottom spot (Fc_1)	0.90	1.00	0.90
Bottom spot (Fc_2)	5.00	2.00	1.29



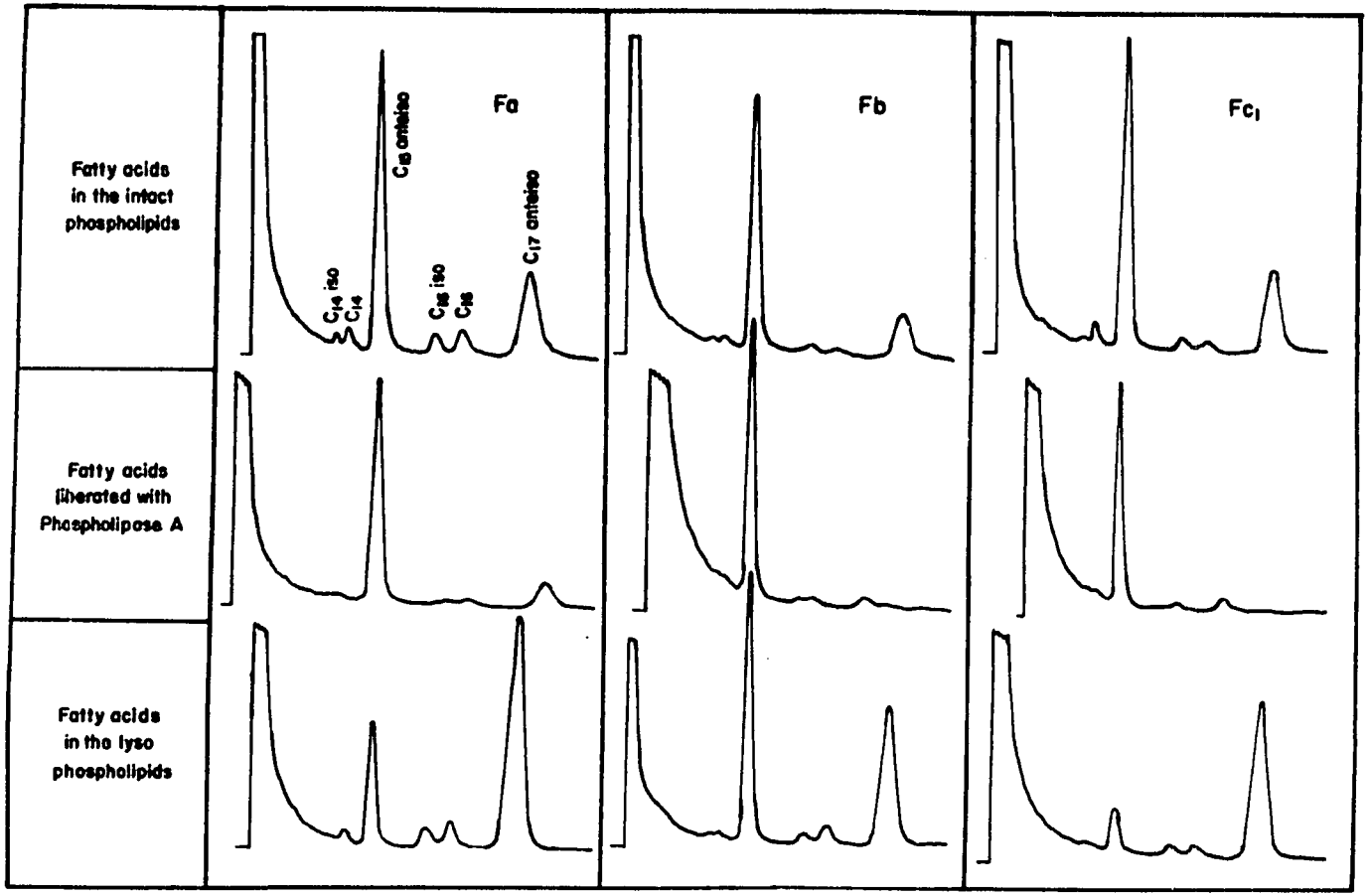
F I G U R E 3 1

Distribution of fatty acids in Fa, Fb and Fc_1

The figure represents the elution pattern of fatty acid methyl esters separated by gas-liquid chromatography. Fatty acids in intact Fa, Fb and Fc_1 are represented. The fatty acids liberated by phospholipase A and the fatty acids in the lyso derivatives are shown as well.

Instrument	- Barber-Colman gas chromatograph, model 5340 with a thermal conductivity cell.
Column	- 3% silicone gum rubber GE SE30 on chromosorb W.
Carrier gas	- helium
Injector temp.	- 220°C
Column temp.	- 185°C
Detector temp.	- 225°C
solvent	- petroleum ether, anal. reagent

DISTRIBUTION OF FATTY ACIDS IN PHOSPHOLIPIDS OF LISTERIA MONOCYTOGENES.

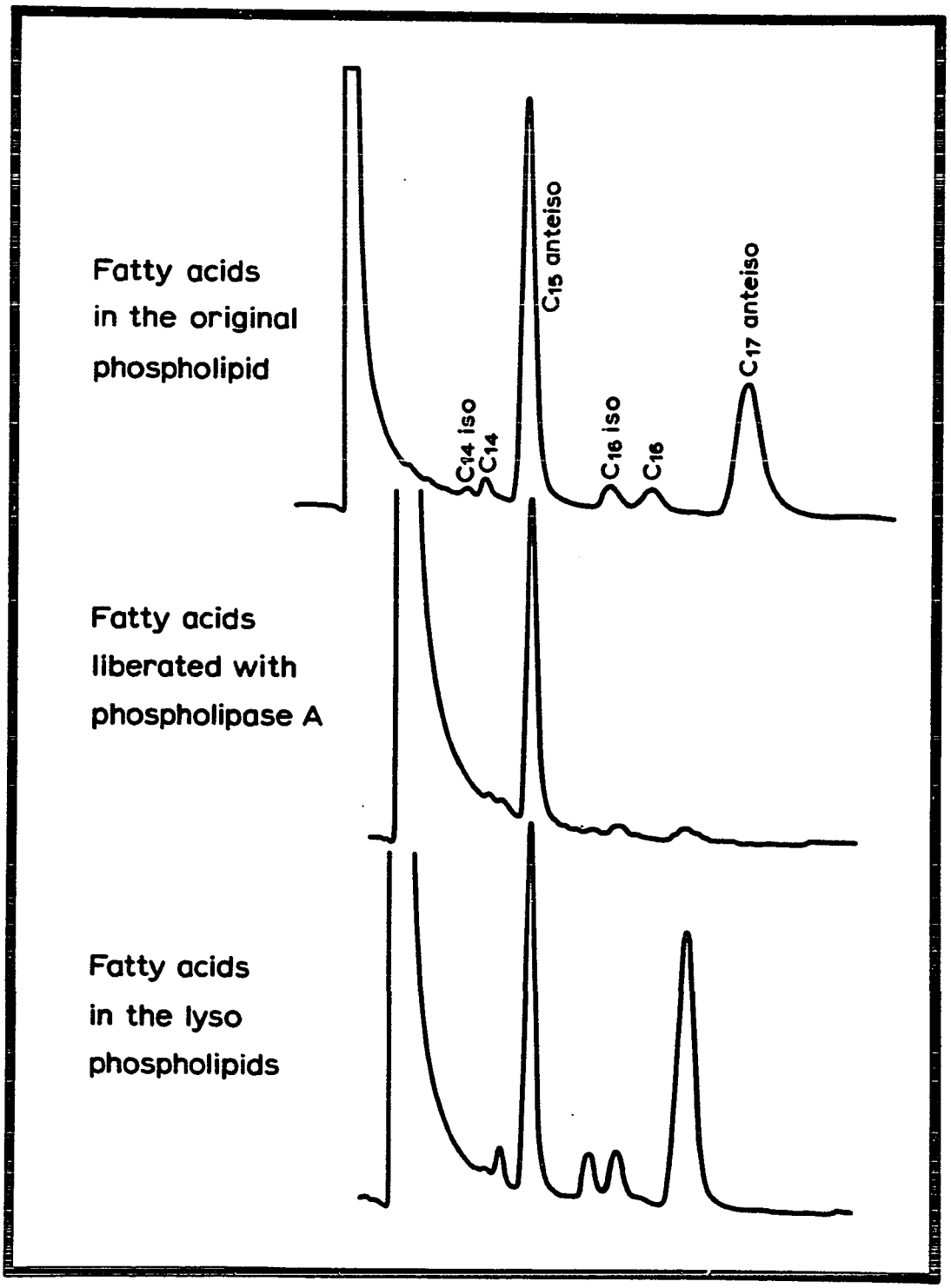


F I G U R E 3 2

Distribution of fatty acids in Fc_2

Chromatograms of the fatty acid methyl esters are presented. Conditions as in Fig. 31.

Distribution of Fatty Acids in Fc_2



are branched-chain C_{15} and C_{17} anteiso acids which vary slightly in their relative proportions in the different phospholipids. Some other acids, such as C_{14} iso, C_{14}, C_{16} iso and C_{16} acids were also found but their quantity was not considerable.

The positional distribution of the fatty acids in the individual phospholipids was investigated by use of gas-liquid chromatography of the fatty acids liberated with phospholipase A as well as by analysis of the fatty acids in the lyso derivatives obtained by treatment with phospholipase A. As seen in the Figures, C_{15} anteiso branched-chain fatty acid (12-methyltetradecanoic acid) was preferentially liberated from all phospholipid samples by treatment with phospholipase A, whereas the C_{17} anteiso acid (14-methylhexadecanoic acid) was predominantly concentrated in the lyso derivative. The other acids showed no significant preference for α- or β-position, except C_{16} acids which showed a tendency to accumulate in the lyso compound.

V. ACETIC ACID HYDROLYSIS

The analytical data obtained for component Fa provided strong evidence that it contained a GPGPG backbone identical with that in beef heart cardiolipin. However, these two compounds were separable in a two-dimensional TLC system, as illustrated in Fig. 17. Both compounds were subjected to hydrolysis with hot acetic acid to investigate whether this difference could be due to a different distribution of fatty acids in the two molecules.

Cardiolipin and Fa were both treated with 90% acetic acid for

TABLE VIII

Fatty acid distribution in phospholipids isolated from Listeria monocytogenes

The relative quantities of the individual fatty acids are presented as % of the total fatty acids found in the investigated sample and they are calculated from the gas-liquid chromatograms of the fatty acid methyl esters by multiplying retention times with the appropriate peak heights.

C_{14} iso - iso tetradecanoic acid (iso myristic acid)

C_{14} - tetradecanoic acid (myristic acid)

C_{15} aiso - 12-methyltetradecanoic acid (C_{15} anteiso fatty acid)

C_{16} iso - iso hexadecanoic acid (iso palmitic acid)

C_{16} - hexadecanoic (palmitic) acid

C_{17} aiso - 14-methylhexadecanoic acid (C_{17} anteiso fatty acid)

GAS LIQUID CHROMATOGRAPHY
(Fatty acids of Listeria phospholipids)

Source	Fatty acids in original phospholipid						Fatty acids liberated with phospholipase A						Fatty acids in Lyso-phospholipid					
	C_{14} ISO	C_{14}	C_{15} AISO	C_{16} ISO	C_{16}	C_{17} AISO	C_{14} ISO	C_{14}	C_{15} AISO	C_{16} ISO	C_{16}	C_{17} AISO	C_{14} ISO	C_{14}	C_{15} AISO	C_{16} ISO	C_{16}	C_{17} AISO
Fa	1·3	2·6	55·4	4·5	6·2	30·0	0·3	0·7	78·0	1·3	2·9	17·0	—	1·3	18·6	4·6	6·3	69·0
Fb	1·2	2·1	68·4	2·3	2·5	23·6	0·9	1·3	86·8	2·3	3·2	5·6	0·3	0·7	45·7	2·5	4·9	45·0
Fc_1	1·0	3·4	58·2	3·5	3·2	30·8	0·8	1·3	85·6	0·7	2·9	8·8	—	0·5	10·2	2·4	3·2	83·7
Fc_2	0·6	2·1	54·8	3·9	4·1	34·5	0·7	1·2	87·5	1·5	3·4	5·6	0·2	2·1	34·4	5·6	6·9	50·8

different periods of time to study the rate of hydrolysis and the nature of the products obtained. After treatment, the lipids were separated from water-soluble products by a Folch-wash procedure and both layers were analyzed by paper and thin-layer chromatography, respectively. The water-soluble products were also analyzed by anion-exchange chromatography.

As reported by Coulon-Morelec et al. (67), glyceroldiphosphate has an R_f value of 0.13 and glycerophosphate an R_f value of 0.37 in an ethanol-water-ammonia system (70:30:2), chromatographed on paper. The water-soluble products from cardiolipin (DPG) and Fa were analyzed in the same system and in both cases, two spots with similar R_f values were observed. The results from anion-exchange chromatography of the water-soluble portion after hydrolysis are represented in Fig. 33. The first peak was eluted at the place expected for glycerophosphate, and the second peak probably represents glyceroldiphosphate, which is expected to be more firmly bound to the ion-exchange resin and therefore eluted with a higher strength of the buffer. The relative quantity of these two compounds is also in agreement with the expected values.

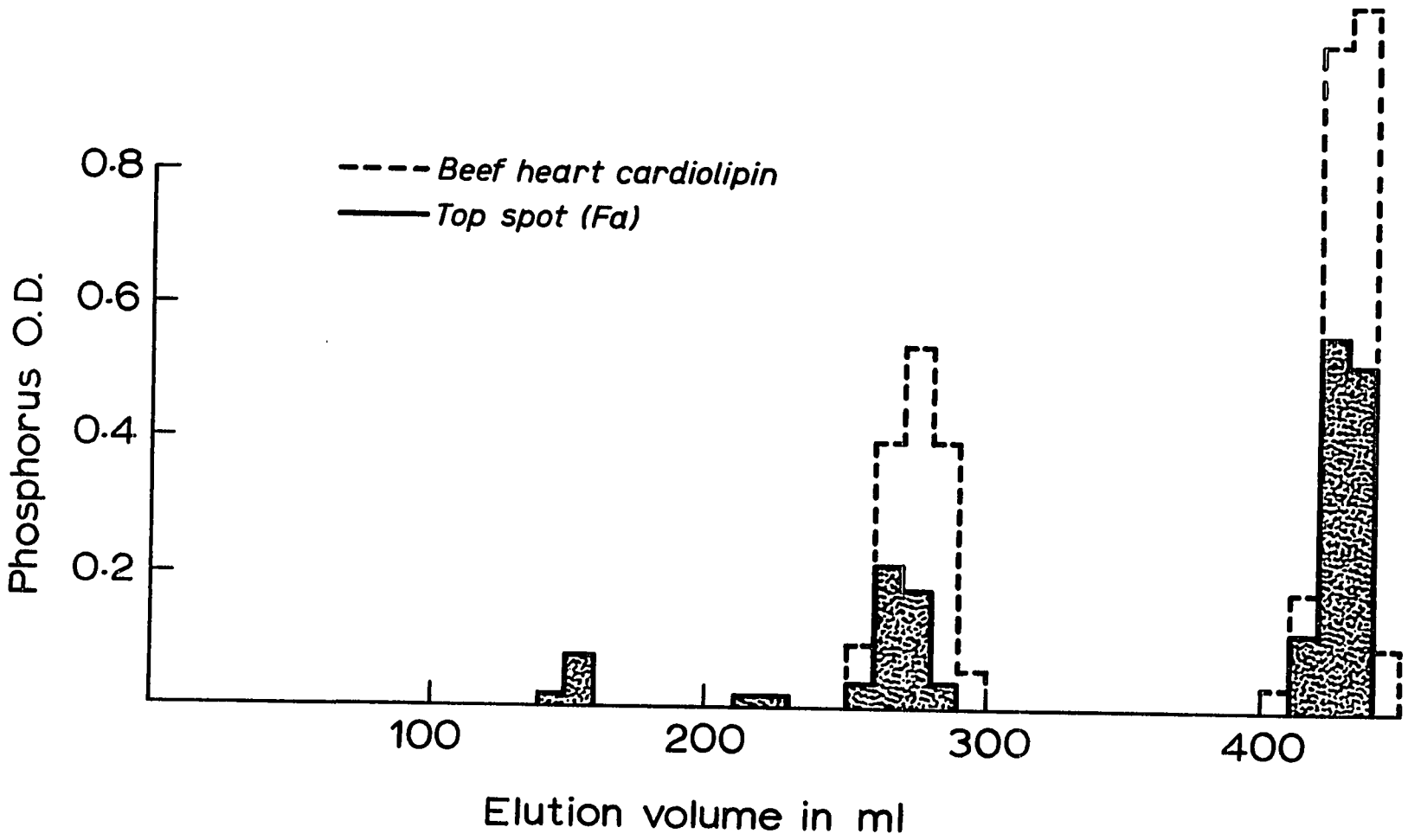
Fig. 34 represents the results obtained by TLC of the $CHCl_3$ soluble portion obtained by Folch-wash from the reaction mixture of cardiolipin and Fa, respectively. The first general conclusion is that the beef heart cardiolipin showed more degradation products than Fa, indicating its inferior stability under these conditions. The main products in both cases, however, were 1, 2- and 1, 3-diglycerides, as expected. The 1, 3-diglycerides are probably formed by isomerization

FIGURE 33

Anion-exchange chromatograms of water soluble products obtained after acetic acid hydrolysis

The chromatograms of the products from cardiolipin standard and Fa are presented. Five ml fractions were collected and individually analyzed for phosphorus content.

ANION EXCHANGE CHROMATOGRAPHY OF WATER SOLUBLE PRODUCTS AFTER ACETIC ACID HYDROLYSIS.



F I G U R E 3 4

TLC chromatograms of the $CHCl_3$-soluble products obtained after acetic acid hydrolysis of cardiolipin standard and Fa

The chromatograms are developed in solvent C. The minutes of treatment with 90% acetic acid at 100°C are indicated.

MG - monoglyceride standard

DG - 1, 3-diglyceride standard

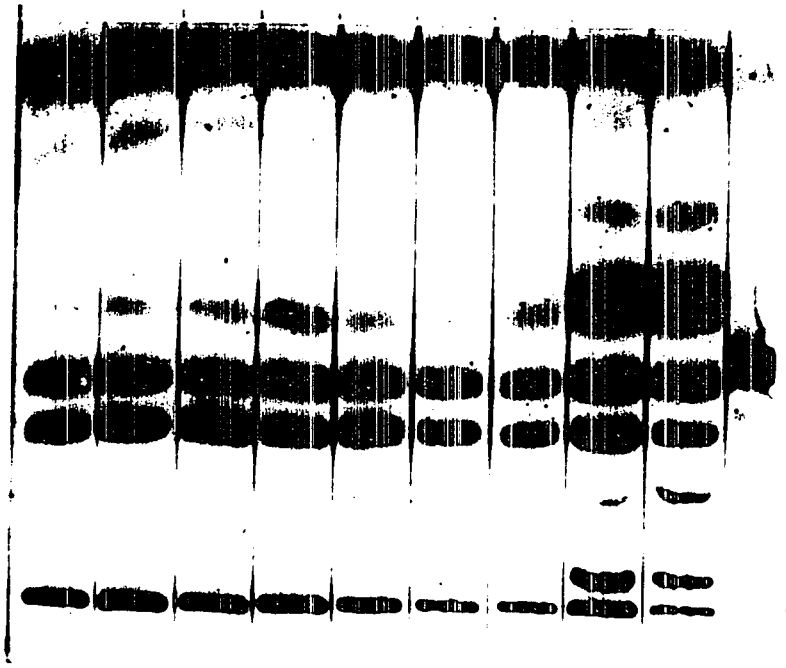
solvent C - petr. ether-ether-acetic acid (60:40:1)

TREATMENT WITH HOT ACETIC ACID

B.H. CARDIOLIPIN

MIN 10 35 60 90 120 150 205 360 MG DG

Fa



10 35 60 90 120 150 205 360 720 DG

PETR. E.-ETH-HAc

of the liberated 1, 2-diglycerides. No more than a trace of monoglyceride was observed in Fa, treated up to 205 minutes with hot acetic acid. However, there are small quantities of free fatty acids present in these samples, running above 1, 3-diglyceride. When Fa was treated for 360 and 720 minutes, a band running at the same place as monoglyceride (MG) was clearly seen and these samples also showed a strong spot representing free fatty acids. Due to the very prolonged treatment (6 and 12 hours), the initially liberated diglyceride was also decomposed, giving rise to monoglyceride and free fatty acids, and probably also free glycerol. The same effect could be observed after hydrolysis of cardiolipin for 360 minutes.

The rates of hydrolysis with acetic acid are represented in Fig. 35, and rates of hydrolysis of Fb, Fc_1 and Fc_2 are also shown in this Figure. As can be seen, Fa is hydrolyzed more slowly than cardiolipin (B.H.Cl.), Fb hydrolyzes with a rate between the two, whereas Fc_1 and Fc_2 show approximately the same rate of hydrolysis as beef heart cardiolipin.

The products obtained after treatment of Fb, Fc_1 and Fc_2 with hot acetic acid are shown in Fig. 36. Products obtained from cardiolipin and Fa are also shown for comparison. In the case of Fb and Fc_1, it is interesting to notice that monoglycerides, as well as free fatty acids, are liberated together with the diglycerides, although the latter again seem to be the most abundant reaction product. Obviously, the degradation of these molecules proceeds by another route.

Another interesting observation was made in the case where

FIGURE 35

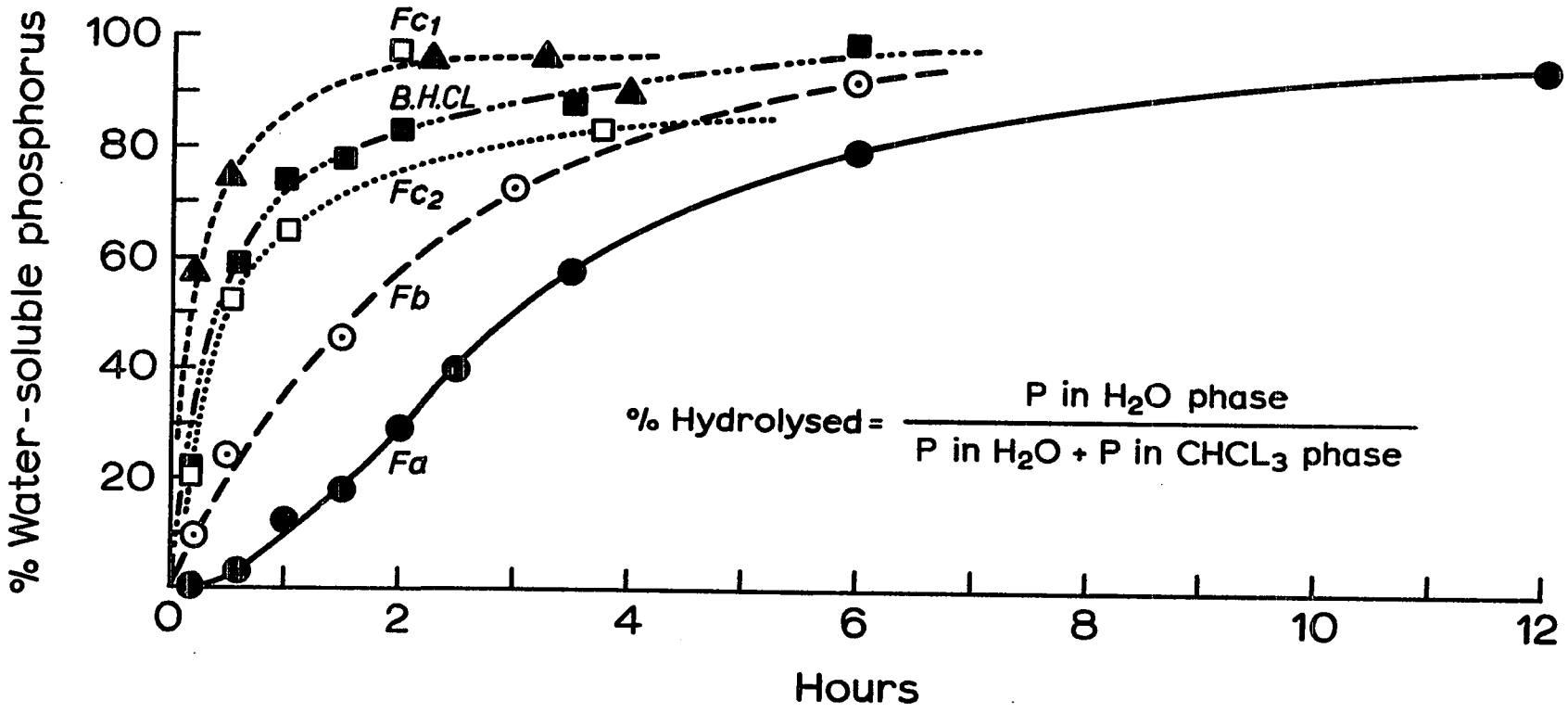
Acetic acid hydrolysis of phospholipids from L. monocytogenes (time study)

Time study of the hydrolysis of isolated phospholipids in hot 90% acetic acid is presented. The % of hydrolysis is calculated according to the formula shown in the Figure.

Fa, Fb, Fc_1, Fc_2 - isolated phospholipids

B.H.Cl. - beef heart cardiolipin standard

HYDROLYSIS OF PHOSPHOLIPIDS FROM L. MONOCYTOGENES.
(90% Acetic acid, 100°C)



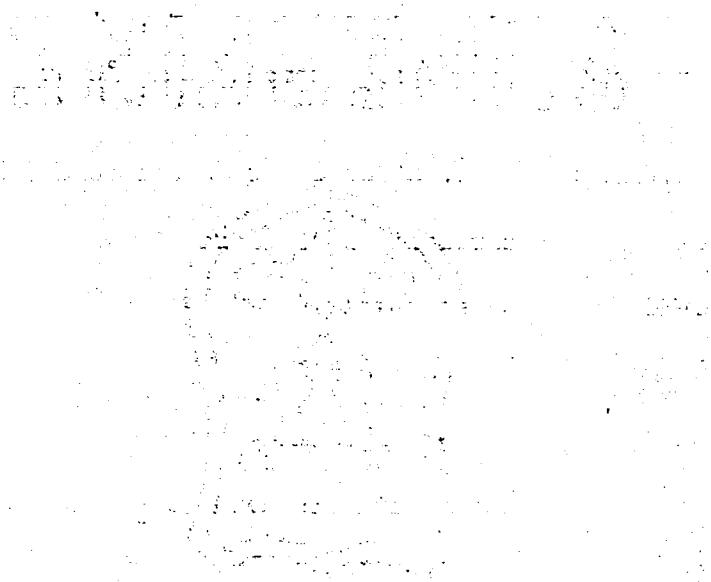


FIGURE 36

TLC chromatograms of the products obtained after acetic acid hydrolysis of Fa, Fb, Fc_1, Fc_2 and cardiolipin standard

The chromatograms are developed in solvent C. The minutes of treatment with 90% acetic acid at 100°C are indicated.

solvent C - petr. ether-ether-acetic acid (60:40:1)

Cl - cardiolipin standard

α-MG - α-monoglyceride

β-MG - β-monoglyceride

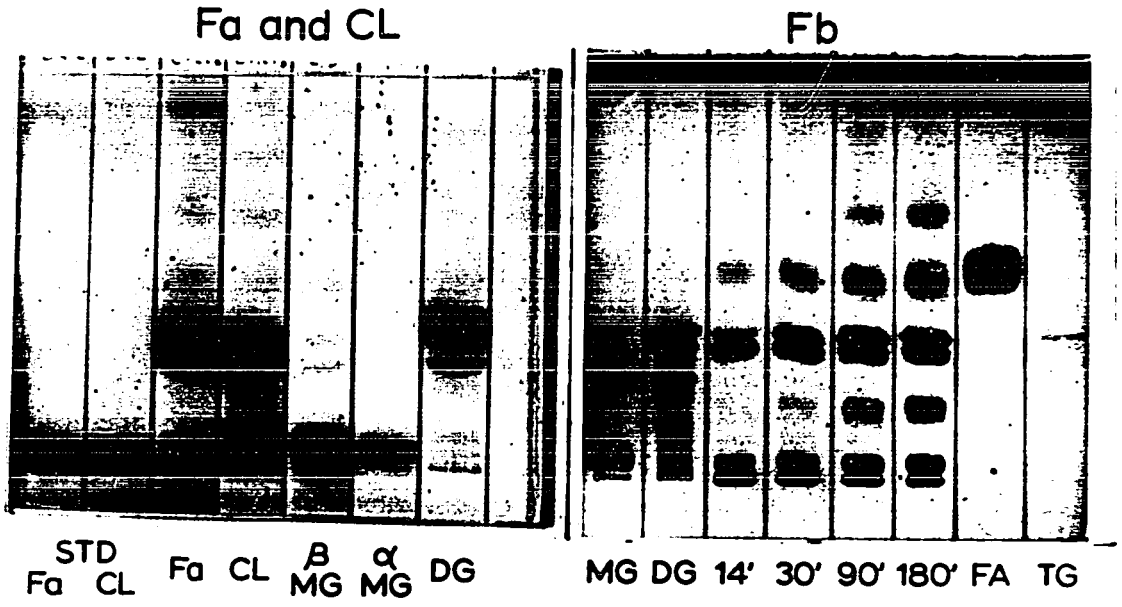
DG - 1, 3-diglyceride

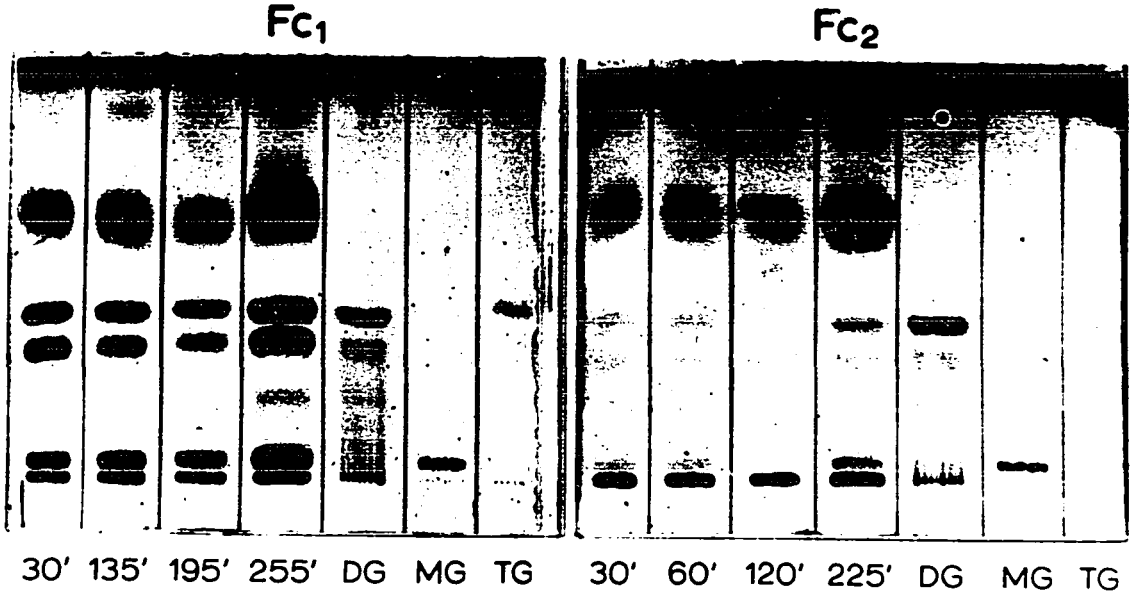
FA - fatty acid standard (C_{15} anteiso acid)

TG - triglyceride

STD - standard

TREATMENT WITH HOT ACETIC ACID.





PETR.E.-ETH-HAC

Fc_2 was treated with hot acetic acid. Whereas the rate of hydrolysis was comparable to that obtained with the other compounds, the final products consisted mainly of free fatty acids rather than diglycerides. This is another of the striking differences between Fc_2 and other phospholipids isolated from Listeria monocytogenes.

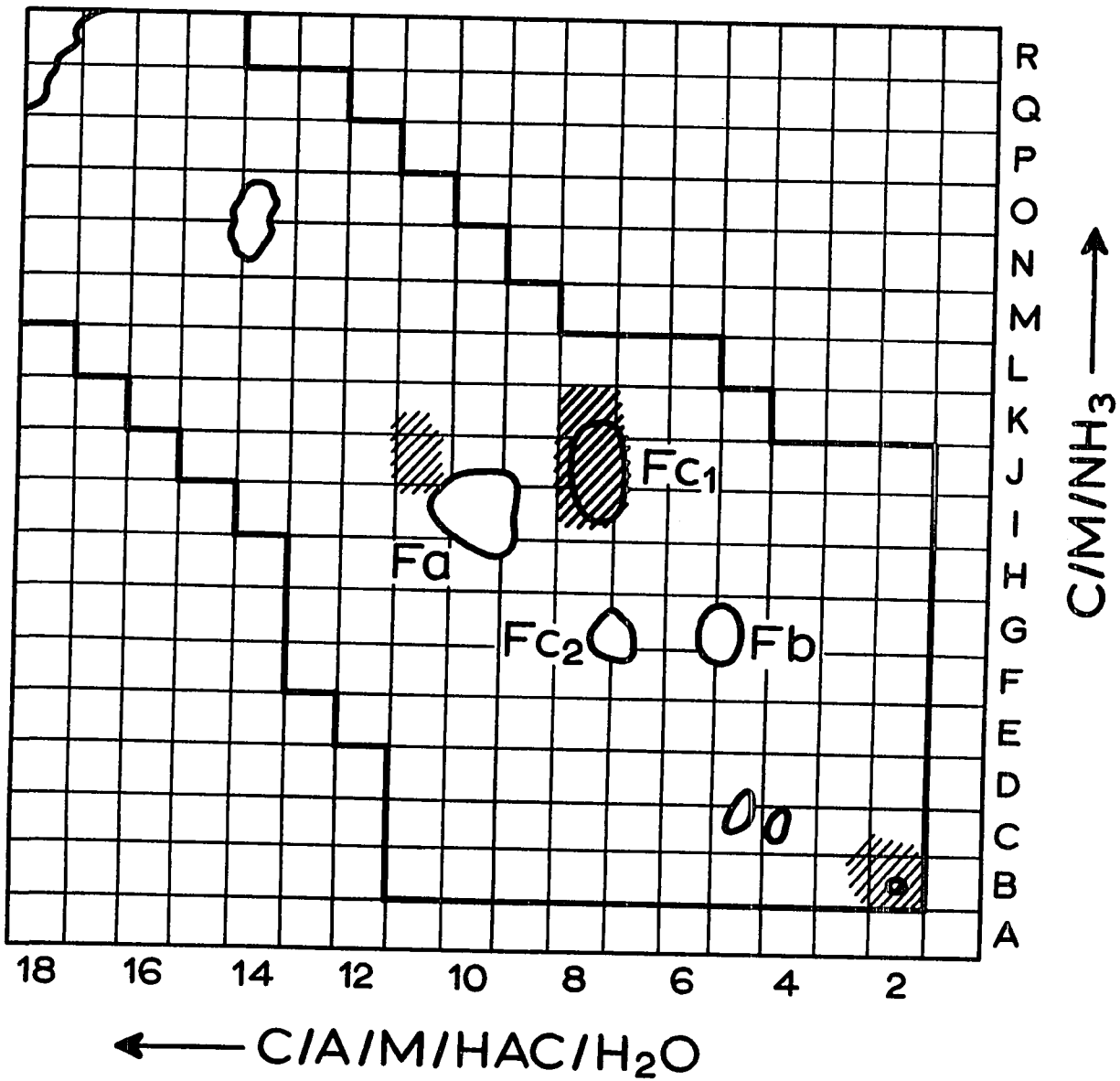
VI EXPERIMENTS WITH LABELLED PHOSPHATIDYLGLYCEROL

Another analysis, which was performed by two-dimensional TLC using a tritium-labelled phosphatidylglycerol, gave additional information on the nature of the Fc_1 compound. This compound was characterized by other methods as a phosphatidylglycerol, and as shown in Fig. 37, it co-chromatographed in the two-dimensional system together with the labelled standard. The location of the activity in different areas of the plate is represented by dashed areas, whereas the open circles represent the places of the corresponding phospholipids analyzed by the same method. The standard contained 6% of labelled diphosphatidylglycerol, and this was detected at the appropriate place on the plate. The activities measured in various portions of the developed TLC plate, are shown in the Table IX.

FIGURE 37

Comparison of a labelled phosphatidylglycerol standard with the phospholipids from L. monocytogenes

A two-dimensional TLC chromatogram is presented. After development, the TLC plate was divided into 324 fields and the middle fields within the thick line were counted for tritium activity. The activity is distributed as indicated by the dashed areas. The open circles represent the location of the corresponding spots. The solvent systems are the same as in Fig. 17.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C/M/NH3
Fc1
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Fc2
Fb
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16
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12
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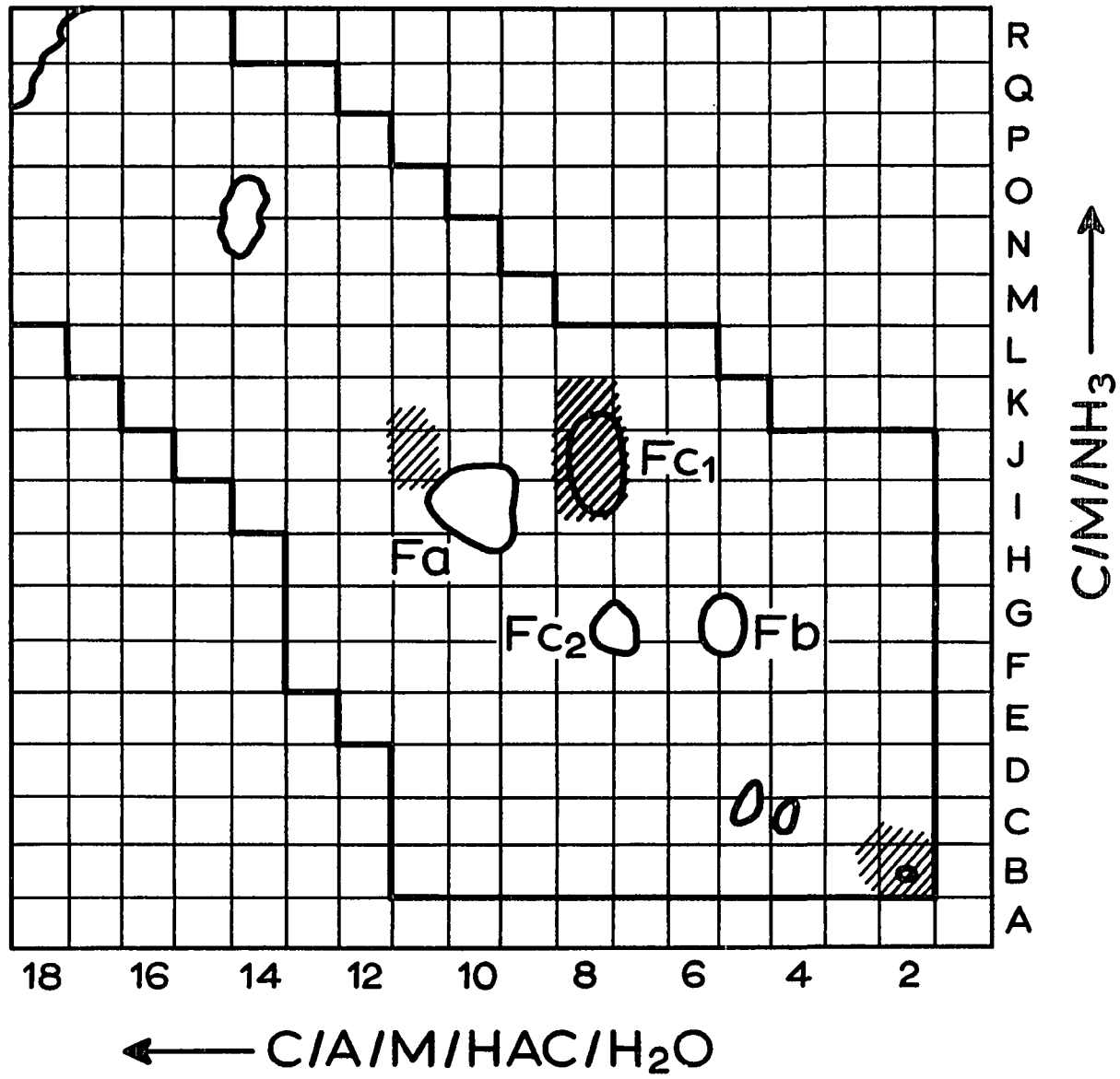
8
6
4
2
C/A/M/HAC/H2O


R
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C/M/NH3
Fc1
Fa
Fc2
Fb
18
16
14
12
10
8
6
4
2
C/A/M/HAC/H2O

TABLE IX

Radioactivity in different areas of the two-dimensional TLC plate used for chromatography of the tritium labelled phosphatidylglycerol

Spot location	Net cpm recorded		
	in sample	total in corresp. area	% of applied material
Applied on plate	870	870	100
2 B	198.8		
3 B	32.3		
2C	37.7		
3C	12.0		
4 C	14.8		
5 C	5.1	300.7	34.5
8 I	34.7		
7 J	11.7		
8 J	182.2		
9 J	6.4		
7 K	15.5		
8 K	176.0		
9 K	6.1	432.7	49.7
11 J	23.0		
10 K	6.6		
11 K	12.0	41.6	4.8
Total	755.0	755.0	89.0

DISCUSSION

The study of phospholipids from Listeria monocytogenes involved a diversified methodology and the combination of various results allowed us to make some propositions about the chemical structure of most of the isolated phospholipids. Some methods which were used in this investigation were taken from the literature but development of new methodology and combinations of different approaches were of great advantage in this study. One of the main problems involved in the study of phospholipid chemistry is associated with the preparation of pure starting materials suitable for further analysis. As phospholipid molecules tend to be unstable under certain conditions (9, 108, 168, 182), degradation during purification procedures has to be kept at minimum values. Degradation can take place during several phases of preparation such as during solvent extraction, chromatography, etc. and side reactions can occur in hydrolytic procedures.

The phospholipid fraction was found to comprise more than 50% of the total lipids extracted from L. monocytogenes. Only a small proportion of the phospholipids was extracted with chloroform alone and most of it was recovered from the chloroform-methanol extract. Although some degradation of the phospholipids may have occurred during storage and extraction of bacteria, degradation products such as diglycerides and free fatty acids would be separated from intact phospholipids by the chromatographic procedures used for purification.

Degradation and isomerization of lipids and phospholipids during chromatography on silicic acid has been observed by many

investigators (35, 45, 121, 272) and similar changes probably occur on acid-treated Florisil, which is essentially a coarse-mesh silicic acid. These effects are particularly noticable in the case of prolonged contact of the lipids with the adsorbent. Traces of impurities may also catalyze the formation of artifacts. Acidic phospholipids are especially unstable during silicic acid chromatography and particularly if they are in the free acid form (106). Corresponding salts of acidic phospholipids, especially the barium salt, are more stable.

It is interesting to mention here some of the results obtained during the course of this study with a beef heart cardiolipin standard supplied as a sodium salt. The cardiolipin sample was chromatographed on an acid-treated Florisil column using the same elution procedure as for Fa. All fractions obtained from the column were combined and subjected to mild alkaline hydrolysis and the water-soluble products were chromatographed by anion-exchange chromatography. The same procedure was carried out with a sample of standard cardiolipin which was not subjected to ATF-chromatography. The results obtained after phosphorus analysis of individual fractions from the anion-exchange column clearly show that there was considerable degradation of beef heart cardiolipin in the sample which was chromatographed on acid-treated Florisil (Fig. 22). However, the phospholipids isolated from L. monocytogenes were not so extensively degraded during the same procedure. The bacterial phospholipids contain mainly branched-chain saturated fatty acids while the cardiolipin contains largely polyunsaturated fatty acids and this may account for the difference

in stability. Rose (223) separated rat liver cardiolipin, containing more than 90% of unsaturated fatty acids, on silicic acid columns and obtained a mixture of five phosphorus containing compounds. Since our experiment with beef heart cardiolipin also gave five phosphorus-containing compounds, the same type of degradation may have occurred in Rose's experiments.

Although side reactions can occur during mild alkaline hydrolysis, an effort was made to minimize these by using conditions specified by Brockerhoff and others (39, 131) as described in the Methods section. Furthermore, the deacylated products were effectively separated from contaminants and side products by anion-exchange and Sephadex chromatography. The anion exchange chromatography was adapted from the work of Wells and Dittmer (268), but the method involving Sephadex chromatography was developed during the coarse of this investigation. This method gave a good separation of the deacylated products from the contaminating salts introduced during mild alkaline hydrolysis and anion-exchange chromatography. As this was a mild procedure and the only solvent for elution was dist. H_2O, no change or degradation of the deacylated phospholipid was expected, and the material was almost 100% recovered from the column. Another advantage of this method is its simplicity and, although the elution procedure lasts for approximately one day, it is not time-consuming, as the fractions are collected automatically on a suitable fraction collector. It is interesting to notice that inorganic phosphate could be also well-separated from the deacylated phospholipid by this method.

Another method of particular advantage in the study of the structure of deacylated phospholipids was NMR spectroscopy. This method is recognized to be one of the most valuable in the study of molecular structure and has found an especially wide application in structural studies of organic compounds (136, 213, 28, 237). Applications of NMR spectroscopy to biochemistry have been reviewed by Jardetzky and Jardetzky (139), and Hopkins (126) has discussed its use in the study of lipids. NMR spectra of some phospholipids and their deacylated products can be found in publications by Chapman and Morrison (55), Serdarevich (233) and others. However, no application of NMR spectroscopy in the study of acidic glycerophosphatides of the cardiolipin type was found in the literature. There is also a very little information available on the NMR spectroscopy of deacylated phospholipids. Only the spectra of α- and β-glycerylphosphorylcholine and α- and β-glycerophosphate were found in the literature (233). One of the reasons for the lack of information in this field may be the difficulty of preparing an adequate amount of pure sample for NMR spectroscopy, and the purification procedure described above was particularly valuable for this purpose.

The NMR spectra obtained, as presented in Figs. 24, 25 and 26 illustrate the value of this type of analysis. Comparing these spectra with those reported in the literature for other deacylated phospholipids (233), one can see that glycerophosphate or polyglycerophosphate is easily recognized by the chemical shifts of the resonating protons, which in all cases resonate in the region of 3.6-4.3 ppm. Any impurity could be detected by noncharacteristic peaks and by peaks appearing at

other frequencies. For instance, the protons on the nitrogen in GPC resonate at 3.2-3.3 ppm (233), giving an indication of the presence of a choline group. If the sample is contaminated with an organic solvent such as methanol, a peak appears at 3.4 ppm, as observed during this study. An interesting observation was also made with a sample which was not quantitatively purified from formate after anion-exchange chromatography. In this case, a sharp peak at very low energy (approx. 8.5 ppm) appeared. This resonance of the formate proton did not influence the spectrum of the deacylated phospholipid, but its appearance could be a good indication of purity of the investigated sample.

The spectra of some original phospholipids such as phosphatidyl-ethanolamine, phosphatidylcholine and phosphatidylserine are reported by Chapman and Morrison (55). These spectra are, however, much more complicated and more difficult to analyze than the spectra of the deacylated phospholipids. This is mostly due to the resonance of the protons associated with the fatty acids present in the former molecules. In this case, the resonance of the protons in $N^{+}(CH_3)_3$ group is shifted to approx. 6.6 ppm. The resonance of the protons on the glycerols esterified to the phosphate group is not distinct and they represent minor broad peaks which are not well resolved. Thus, the NMR spectra of intact phospholipids do not give conclusive evidence about the arrangement of the protons on the corresponding glycerols. The most prominent peaks in these spectra are associated with the protons of the fatty acids.

The analytical procedures and their interpretation deserve some

comment as well. It is of interest to notice that most of the work in the past, concerning the structure of various phospholipids, and especially acidic phospholipids has been based on molar ratios of the individual groups present in the molecule (85, 93, 192, 204). This is particularly true in the case of cardiolipin. Its structure was proposed by Pangborn on this basis and the strongest evidence for an alternate diphosphatidylglycerol structure was supported by molar ratios also. However, one should be aware of the possibility of an erroneous interpretation of the results obtained by chemical analysis. The analytical methods which are applied in such analyses are in almost all cases micro- or semimicro methods and are, therefore, more subject to error. Particular care is necessary in applying such methods which are, a priori, known to comprise large experimental errors. One of them, for example, is the determination of glycerol. As noticed by many investigators, the determination of glycerol in a phospholipid molecule is a very difficult procedure (108, 9). The most accurate methods involve an acid hydrolysis of the phospholipid molecule with liberation of free glycerol which is then determined as formaldehyde by periodate oxidation (68, 219). However, it is noticed that during hydrolysis of the glycerol-phosphate linkage, a considerable amount of glycerol can be degraded, resulting in low glycerol values (9, 68, 108, 109) and the amount of degradation may be different for different glycerophosphates (109). Since the glycerol-phosphate bond is difficult to hydrolyze, another error can arise from non-quantitative liberation of free glycerol. Inclusion of a glycerol standard eliminates, or rather diminishes some of these errors.

I. PROPOSED AND POSSIBLE STRUCTURES FOR PHOSPHOLIPIDS ISOLATED FROM L. MONOCYTOGENES

The phospholipids isolated from Listeria monocytogenes appeared to be composed essentially of three structural units: glycerol, phosphate and fatty acids and did not seem to contain other components such as amino groups or choline, sugars or inositol. The molar ratios of these units in the intact and deacylated phospholipids are summarized in Table X and the arrangement of these components in the different phospholipids will now be considered.

A. Top spot (Fa)

This compound, which had the highest R_f value on TLC (solvent A), showed an approximate molar ratio of ester:phosphorus:glycol:glycerol of 4:2:0:3. Its lyso derivative had an ester:phosphorus:glycol ratio of 2:2:0 and the deacylated product had 2 moles of phosphorus per 2 moles of glycol. The deacylated product had the same R_f value as deacylated beef heart cardiolipin, as determined by paper chromatography in 5 different solvent systems. These two compounds also had the same elution pattern on anion-exchange chromatography. The IR spectrum of Fa was almost identical with the spectrum of cardiolipin and the NMR spectra of the deacylated products were shown to be undistinguishable. In both cases, the same products were obtained by acetic acid hydrolysis. It has been clearly established that beef heart cardiolipin has a diphosphatidylglycerol structure (106). This structure fits our analytical data for Fa and it is, therefore,

TABLE X

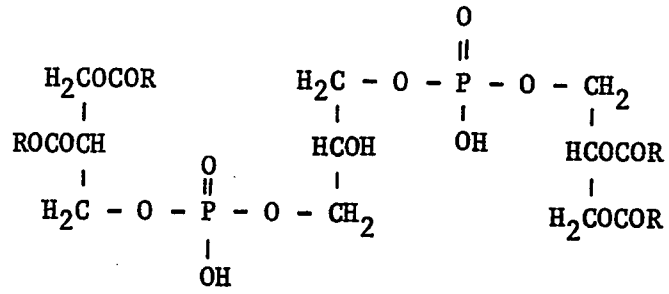
Chemical analysis of intact and deacylated phospholipids

The molar ratios, relative to phosphorus, for Fa, Fb, Fc_1 and Fc_2 and their derivatives, are illustrated. The ratios obtained with the deacylated beef heart cardiolipin standard are also included. The values are rounded to the first decimal.

ANALYSIS OF PHOSPHOLIPIDS FROM LISTERIA MONOCYTOGENES.
(Molar ratios).

Compound	Original phospholipid				Lyso derivative			Deacylated product	
	Ester	Phosph.	Glycol	Glycerol	Ester	P	Glycol	P	Glycol
Cardiolipin standard	—	—	—	—	—	—	—	2·0	2·0
Fa	4·2	2·0	0·0	2·8	2·2	2·0	0·0	2·0	2·0
Fb	3·8	3·0	0·0	4·0	1·9	3·0	0·0	3·0	2·0
Fc_1	2·1	1·0	0·9	1·8	0·9	1·0	0·9	1·0	2·0
Fc_2	6·0	2·0	0·4	3·9	5·0	2·0	1·3	2·0	3·4

concluded that this phospholipid has the same basic structure as beef heart cardiolipin, as illustrated below.



Diphosphatidylglycerol (DPG)

The only discrepancy in the results obtained with these two compounds was a difference in mobility when they were chromatographed on a two-dimensional TLC plate (Fig. 17). The reason for this separation was investigated and three possibilities were envisaged:

1. Different salt forms.
2. Different distribution of fatty acids.
3. Difference in the nature of the fatty acids.

It was reported by de Haas, Bonsen and van Deenen (106) that different salt forms of the same phospholipid can have different chromatographic characteristics. To investigate whether this effect could account for the difference in mobility of the two compounds on a TLC plate, both cardiolipin and Fa were treated with 0.1N HCl to convert the lipids into the free acid form but these forms were still separable from one another.

To see whether the fatty acids in Fa are esterified only on the terminal glycerols, as in the case of DPG, both cardiolipin and Fa were treated with 90% acetic acid at 100°C and the products were

compared by TLC, paper and anion exchange chromatography. The mechanism of this degradation, as reported by Coulon-Morelec, Faure and Marechal (67), is represented in Fig. 38. When a DPG molecule is treated with hot acetic acid, hydrolysis of phosphate ester bonds takes place with the formation of a cyclic intermediate and appropriate isomerizations. The first step involves a preferential hydrolysis of bond 1 with liberation of a diglyceride and a compound with a cyclic phosphate ester. Further degradation of the latter compound can be either by route A or B. As found by Coulon-Morelec and his associates, route A is followed preferentially in hydrated acetic acid and route B in glacial acetic acid. Following route A, the first step would be an opening of the cyclic phosphate ring and, as bond 1' in the resulting compound would be more susceptible to hydrolysis, another diglyceride is liberated as well as the resulting cyclic ester. Opening of this ester yields a glyceroldiphosphate, as indicated.

Route B involves a splitting of the 2' bond, producing a phosphatidic acid and another cyclic ester, which finally opens and gives glycerophosphate. In this way, if the molecule has a DPG structure, the final products expected are diglycerides, glycerophosphate and glyceroldiphosphate. Using aqueous acetic acid, more glyceroldiphosphate than glycerophosphate is expected. As seen in this scheme and stated by the authors, no free fatty acids are expected by this treatment.

Since Fa and cardiolipin both gave predominantly diglycerides, with little or no monoglyceride (Fig. 34) as expected from the hydrolytic pathway for DPG and, since both gave the same water-soluble

F I G U R E 3 8

Degradation of diphosphatidylglycerol with hot acetic acid

A scheme presenting the pathway during acetic acid degradation of DPG is illustrated.

DPG - diphosphatidylglycerol

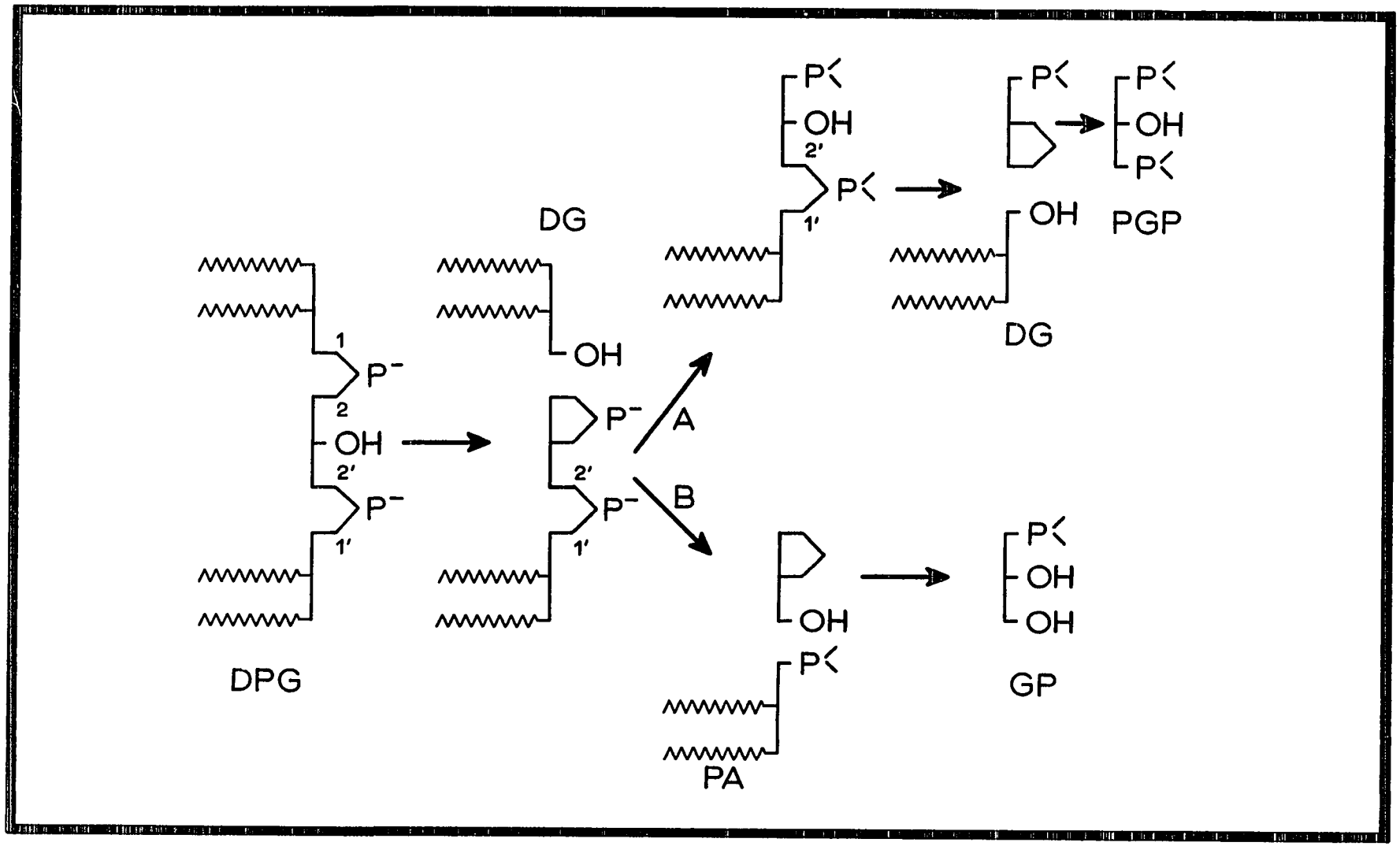
DG - diglyceride

PA - phosphatidic acid

GP - glycerophosphate

PGP - glyceroldiphosphate

Degradation of Diphosphatidylglycerol with Hot Acetic Acid.

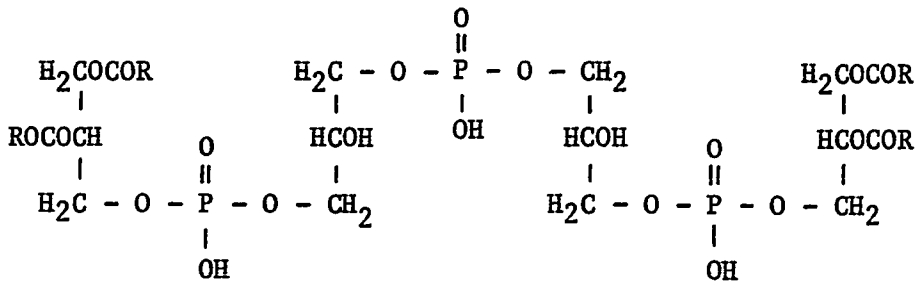


hydrolytic products (Fig. 33), it seems very probable that the fatty acids are esterified to the terminal glycerols in each case.

It was, therefore, concluded that the different mobilities of cardiolipin and Fa on a two-dimensional TLC plate were due to the known differences in fatty acid composition of these two compounds. Several investigators have observed different mobilities of lipids during silicic acid chromatography as a result of differences in unsaturation of the constituent fatty acids. Rhodes and Lea (220) noticed that the more unsaturated molecules of phosphatidylcholine were enriched in the front of the lecithin peak during chromatography on silicic acid with $CHCl_3$-MeOH. Similar observations were reported by Harris et al. (112) and the higher mobility of the more unsaturated phosphatides on silicic acid was also noticed by Renkonen (218).

B. Middle spot (Fb)

The compound with an R_f value somewhat lower than Fa (in solvent A) showed an approximate molar ratio of ester:phosphorus:glycol: glycerol of 4:3:0:4 (Table X). The lyso derivative showed an ester: phosphorus:glycol ratio of 2:3:0 and the deacylated product had 3 phosphates per 2 glycol groups. The infrared spectrum of Fb was almost identical with that of Fa. The deacylated compounds from Fa and Fb could be separated by paper chromatography as well as by anion-exchange chromatography. Their NMR spectra also showed differences in peak areas and chemical shifts. The results of these analytical procedures are in agreement with a bis-phosphatidylglyceryl phosphate structure for Fb as presented on the following page:



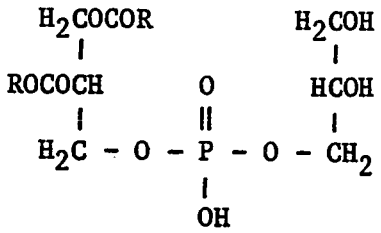
bis-phosphatidylglyceryl phosphate

This structure is similar to that proposed for Fa but contains one more glycerophosphate in the molecule. It has the same backbone as the structure originally proposed by Pangborn for cardiolipin (204). However, component Fb is formulated with fatty acids esterified only on the terminal glycerols, whereas the Pangborn structure had six fatty acids and no free -OH groups in the central glycerols. To our knowledge, no compound with this structure has previously been identified in living cells.

C. Bottom spot (Fc_1)

The Fc component obtained by preparative TLC in solvent A was a mixture of two compounds which could be separated by TLC in solvent B. The one with the larger R_f in solvent B was designated as Fc_1. This compound had an approximate molar ratio of 2:1:1:2 for ester: phosphorus:glycol:glycerol. The lyso derivative showed an ester: phosphorus:glycol ratio of 1:1:1, whereas the deacylated product had a molar ratio of 1 phosphate per 2 glycol groups. Fc_1 had the same mobility as a labelled sample of phosphatidylglycerol standard in a two dimensional TLC system and its infrared spectrum was almost

identical with those of Fa and Fb. The deacylated product from Fc_1 was separated by paper chromatography from corresponding products of Fa and Fb. Furthermore, it was eluted from an anion-exchange column at the place where glycerylphosphorylglycerol is expected and its NMR spectrum provided additional evidence in favour of this structure. On the basis of these results, it is proposed that Fc_1 is a phosphatidylglycerol of the type represented below:



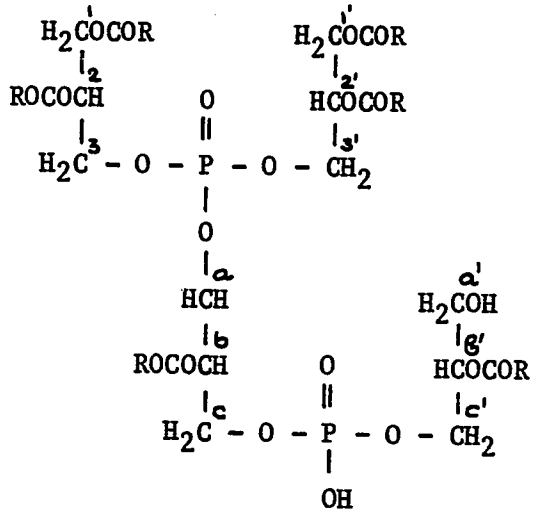
phosphatidylglycerol

D. Bottom spot (Fc_2)

This compound was not separable from Fc_1 by TLC in solvent A, but was separated in solvent B where it had a lower R_f value (Fig. 16). The intact phospholipid had an approximate ester:phosphorus:glycol: glycerol ratio of 6:2:0:4 (Table X). Its lyso derivative had a ratio of ester:phosphorus:glycol of 5:2:1 and the deacylated product showed a ratio of phosphorus:glycol of 2:3. The compound showed approximately the same rate of hydrolysis in 0.05N NaOH and in 90% acetic acid as the other three phospholipids and the deacylated products of Fc_1 and Fc_2 were eluted together from the anion-exchange column. However, the deacylated product of Fc_2 was separable from the corresponding products of Fa, Fb and Fc_1 by paper chromatography and it appeared as a single

spot in all solvents tested. The infrared spectrum of the intact phospholipid was different from the others (Fig. 19). The NMR spectrum of the deacylated product was also rather different from the spectra of the other compounds, but the chemical shifts indicated a phospholipid molecule.

It was difficult to conceive of a structure comprising only glycerol, phosphate and fatty acids which would accommodate these data. The observed molar ratios in the original phospholipid and its deacylated and lyso derivatives are particularly hard to explain. However, such molar ratios are possible with a molecule containing a phosphate triester as illustrated below:



This structure, in fact, represents a dimer of the Fc_1 backbone, in which one of the glycerylphosphorylglycerols is connected to the other in such a way as to form a phosphate triester.

It is, however, still an open question whether phosphate triesters exist among natural phospholipids. There are numerous

reports in the literature which favour a phosphate triester structure of phospholipids and these come mainly from two laboratories--those of Collins and Galanos. Collins and his associates have published a series of papers (62, 63, 64, 65) suggesting the existence of "complex phospholipids" which contain phosphate triester groups. The evidence obtained for such lipids was based on molecular weight of the compounds investigated, as well as on the countercurrent distribution of the diphenylated and methylated lipids. The molecular weight was found to be at least double that of the known phospholipids. They found about 39% of such complex lipids in the total lipids of rat liver, 28% in sheep brain, 41% in rat heart, 28% in influenza virus, 10% in cabbage leaves, etc. According to these findings, Collins and his associates have suggested that the majority of native lipids are in the form of phosphate triesters which are split during hydrolytic procedures applied for investigation of their structure. The resulting lipids, which are characterized in such hydrolysates, are only split products arising from more complex lipid structures which exist in the cell. However, the statement about instability of phosphate triesters, as made by Collins, is not supported by chemical data as phosphate triesters are reported to be relatively stable compounds (41, 212).

Galanos and Kapoulas are very much in favour of the suggestions presented by Collins and have published several papers on the phosphate triester structure of phospholipids (88, 89, 90). These authors have reported the isolation of complex lipids by mild fractionation procedures such as countercurrent distribution and suggest that triester phospholipids, in general, comprise the precursors of commonly

known phospholipids, and that "they are probably the native compounds of most animal and plant tissues". However, to give such a broad generalization in the present stage of phospholipid chemistry is rather unjustified as there must be more supporting evidence accumulated before such a statement can be made. The possibility of the existence of triphosphate esters should not be excluded a priori and it will be interesting to see whether this "unorthodox view of tissue phospholipids" (9) will be supported by more chemical evidence in the future.

It can be seen that the molar ratios obtained for Fc_2 and its deacylated and lyso derivatives are in agreement with the phosphate triester represented above. Hydrolysis of Fc_2 with phospholipase A liberates only one fatty acid out of six, producing at the same time one glycol group and this result could also be explained by the above structure. According to the specificity of phospholipase A and the minimum structural requirements for a phospholipid molecule to serve as substrate for phospholipase A (Fig. 5), only the fatty acid on position b' fulfils these requirements and when it is hydrolyzed from the molecule, a free glycol group results. The fatty acids which are distributed on the β-position of other glycerols would not be hydrolyzed since there is no free hydroxyl function on the adjacent phosphate. The fatty acids on the b position could be considered as being in a sterically inconvenient position for attack by the enzyme and this could explain why that particular ester bond is not hydrolyzed.

The NMR spectrum (Fig. 26) also provides some support for the structure above. The resonance occurs in the same area as in the case

of other deacylated phospholipids investigated, but it does not give conclusive evidence of the identity of Fc_2 and the triester. However, the spectrum could fit the structure illustrated. The protons resonating at the highest field could be the ones on carbons 1, 1' and a', the protons on carbons 2, 2' and b' could be represented by the peak at the lower field, whereas the protons on carbons 3, 3' and c could resonate at the still lower field at approx. 2650 Hz. The protons on carbons a and b, which are under the strong influence of both phosphate groups would be expected to resonate at the lowest field (approx. 2660 and 2670 Hz). As the spectrum is very complex, such an analysis presents only a rough approximation.

Although the results presented above are in favour of the triester structure for Fc_2, other considerations argue against such a structure. The placing of a fatty acid at position b', which was necessary to explain the results with phospholipase A, is rather unlikely since it is known that fatty acids in the β-position of phospholipids tend to isomerize to the α-position. Another problem relates to the products of acetic acid hydrolysis of Fc_2. Fa gave by this hydrolysis mainly diglycerides in accordance with the proposed mechanism (Fig. 38), whereas Fc_2 gave mainly free fatty acids with very little diglyceride (Fig. 36). However, it may be noted that Fb and Fc_1 gave both free fatty acids and monoglycerides in addition to diglycerides, which was also not expected according to the mechanism for DPG. There are obviously other factors which influence the degradation of phospholipid molecules in the coarse of acetic acid hydrolysis and their investigation would be of interest.

Structural analysis of Fc_2 is also complicated by the consumption of periodic acid in amounts slightly in excess of unit molar values in the coarse of glycol determinations (Table X). It is possible that Fc_2 is actually a mixture, although the deacylation product gave a single spot in five different paper chromatographic systems. A second small peak, corresponding to α-GP was, however, observed on anion-exchange chromatography of the deacylated Fc_2 (Fig. 21). Fc_2 appeared to consist entirely of glycerol, phosphate and fatty acids, but there may be other factors which were overlooked and which could account for the difficulty in assigning a structure to this compound as well.

E. Positional distribution of fatty acids

The backbone of three of the investigated phospholipids as well as the overall position of the fatty acids in these molecules has been established. However, it was of interest to know whether the individual acids were randomly distributed in the molecules or whether they were specifically esterified to the α- or β-position of the glycerols. This was determined by treatment of all intact phospholipids with phospholipase A, and this was also one of the contributions made by this study.

The overall composition of fatty acids in L. monocytogenes was reported (49, 214). The composition of fatty acids in different phospholipids was determined in this study and it is in agreement with the previously reported data (49). However, there was no report in the literature on the positional distribution of fatty acids in the

various phospholipids of Listeria monocytogenes. As found in this study, the fatty acids are not randomly distributed. The C_{15} anteiso acid occupies preferentially the β-position on glycerol, whereas the C_{17} anteiso acid was found to be predominantly esterified to the α-position. However, as seen in Table VIII, there is always some C_{17} anteiso acid present in the acids liberated with phospholipase A and there are some C_{15} acids in the lyso derivatives, which could account for different molecular species among these phospholipids. The results obtained by this investigation are in agreement with the general idea that shorter chain fatty acids and unsaturated acids tend to occupy the β-position, whereas longer fatty acids and more saturated ones preferentially esterify the α-position of the glycerols (40, 120, 200, 201).

II. BIOLOGICAL ACTIVITY

Although the study of the phospholipids in Listeria monocytogenes was primarily undertaken because of the biological activity which seemed to be associated with these lipids, no specific compound was isolated to which the biological activity could be ascribed. When tested in animals, all phospholipid fractions showed some activity, but this seemed to be mostly associated with the components having lower R_f values on TLC in solvent A.

While the present studies on phospholipid structure were in progress, other experiments being carried out in this laboratory provided evidence that the monocyte-producing agent (MPA) is a water-soluble substance associated with the phospholipids (R. A. Tadayon and

K. K. Carroll, unpublished data). Several investigators have observed that lipids, and especially phospholipids, solubilize into organic solvents many non-lipid materials such as inorganic salts, proteins, amino acids, sugars, water soluble phosphate esters, etc. (9, 17, 108, 182). This effect is partly due to the solubilization of these substances in lipid solvents but it is mostly due to complex formation and such complexes may not be easily dissociated.

SUMMARY AND CONCLUSIONS

The phospholipids of Listeria monocytogenes were shown to be composed of three main components: glycerol, phosphate and fatty acids. They were isolated from bacterial cells by $CHCl_3$ and $CHCl_3$-MeOH extraction and separated from non-polar components and from glycolipid by acid-treated Florisil column chromatography. The individual phospholipids were separated by preparative thin-layer chromatography in two different solvent systems. Four main components were obtained by this separation procedure and were further purified and analyzed. The individual phospholipids were analyzed for phosphorus, ester, glycerol and glycol groups and were subjected to enzymatic degradation in order to analyze the products and to study the fatty acid distribution in the molecules. The original phospholipids were also analyzed by infrared spectroscopy and by one- and two-dimensional thin-layer chromatography.

In order to determine the molecular structure of the individual phospholipids, they were deacylated with mild alkali and the deacylated products were separated by anion-exchange and Sephadex chromatography. The pure products obtained by this treatment were analyzed for phosphorus and glycol groups. They were also analyzed by paper chromatography and NMR spectroscopy. Additional information on the structure of the four components was obtained by analysis of the lyso derivatives, of the products obtained by acetic acid hydrolysis and by comparison with authentic samples.

The results can be summarized as follows:

All four components are polyglycerophosphatides with different proportions of phosphate, glycerol and fatty acids in the molecule. Three of the phospholipids are similar and the following structures are suggested:

Isolated lipid	Proposed structure	% of total phospholipids
top spot (Fa)	diphosphatidylglycerol	45-50
middle spot (Fb)	bis-phosphatidylglyceryl phosphate	8-10
bottom spot (Fc_1)	phosphatidylglycerol	40-45

The fourth component (Fc_2) which comprised 5-7% was shown to be different in a number of respects from the other three and it was not possible with the data obtained to suggest a structure for this compound, although the possibility of a phosphate triester was considered.

Fatty acids in all four components are non-randomly distributed, C_{15} anteiso acid being esterified to the β- and the C_{17} anteiso acid to the α-position of the glycerols.

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